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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 235, 479–481 (1997)
ARTICLE NO. RC976806

A Putative Tetrapeptide Antagonist Prevents β-Amyloid-Induced Long-Term Elevation of $[Ca^{2+}]_i$ in Rat Astrocytes

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Received May 15, 1997

Comparative fluorimetric studies on the long-term (8-hour) action of β[1-42]amyloid and its shorter fragments β[1-40], β[25-35] and β[31-35] on the steady-state intracellular Ca^{2+} concentration in primary cultures of rat astroglial cells using the Ca^{2+}-sensitive fluorescent probe Fura-2 AM revealed higher 340/380 fluorescence excitation ratios in the treated cells as compared to the untreated controls. All the peptides were found to induce similar cellular effects, suggesting the [31-35] region as the putative active centre of the molecule. No significant alteration was detectable in Fura-2 fluorescence using the Ca^{2+}-insensitive excitation wavelength of 367 nm, indicating that the observed changes reflect a real alteration in the Ca^{2+} concentration of the cells. Moreover, no considerable difference was observed in the total protein content of treated and untreated cells. Co-treatment of the cells with Pr-Ile-Ile-Gly-Leu-NH_2 (Pr-IIGL) peptide, an analogue of the [31-34] region of β[1-42]-amyloid, was found to effectively antagonize the β[1-42]-amyloid-induced elevation of the fluorescence excitation ratio, leaving the 367-nm fluorescence unaffected. To the best of the authors' knowledge, this is the first report on an analogue of β-amyloid peptide capable of blocking one of its physiological effects, thereby raising the possibility that this sequence could prove to be a lead compound for designing effective β-amyloid antagonists.

Excessive accumulation of β[1-42]-amyloid peptide in the brain is a major characteristic of Alzheimer's disease. This peptide, produced from the ubiquitous membrane-protein amyloid precursor protein (APP) by a pathogenic 'alternative' cleavage process (1-2), has been shown to induce cytotoxic actions in neural and glial cells leading to cell degeneration both *in vitro* (3) and *in vivo* (4). Concerning its primary cellular effects, elevation of protein phosphorylation (5), modification of cytosolic Ca^{2+} homeostasis (6), and production of O2 free radicals (7) have been reported in neural cells. More recently, β-amyloid-induced production of O_2 free radicals was observed in vascular endothelial cells (8). Although all of these observed physiological alterations have been implicated in the development of cytotoxic symptoms in neural cells, we know very little about the connection between the early effects and the onset of cellular degeneration. In this communication, we report on the synthesis of β[1-42]amyloid and its shorter fragments β[1-40], β[25-35], β[31-35] peptides and present results demonstrating that the tetrapeptide Pr-Ile-Ile-Gly-Leu-NH2 is capable of blocking the long-term cellular effect of β[1-42]-amyloid.

MATERIALS AND METHODS

Synthesis of amyloid peptides. All amino acid derivatives, solvents, chemicals, and the resins were obtained commercially and used without further purification. Protected amino acids used in the syntheses were of the L-configuration. The α-amino function was protected with *tert*-butoxycarbonyl (Boc) group, and the reactive side chain functional groups were protected as follows: *para*-toluene-sulfonyl for Arg; cyclohexyl for Asp and Glu; benzyloxycarbonyl for His; 2-chlorobenzyloxycarbonyl for Lys; benzyl for Ser; and 2-bromobenzyloxycarbonyl for Tyr. The side chains of Asn and Gln were unprotected. For peptides with an amidated carboxy-terminus, *para*-methylbenzhydrylamine (MBHA) resin (0.57 mmol/g) and for peptides with free carboxy-terminus Merrifield resin (1 mmol/g) was used.

The amyloid peptides were prepared by solid phase methodology (9) using manual solid phase peptide synthesis equipment for shorter peptides and ABI 430A automated peptide synthesizer for longer peptides. The coupling reactions were achieved with a 3-fold excess of Boc-amino acids using DCC or DCC/HOBt as activating agent in dichloromethane, dimethylformamide or mixtures thereof. Boc-Asn and Boc-Gln were coupled with preformed 1-hydroxybenzotriazol ester to avoid side reactions. After a coupling time of 2 hour, th

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Abbreviations used: $[Ca^{2+}]i$, intracellular free Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; Fura-2-AM, acetoxymethyl ester derivative of Fura-2; GFAP, glial fibrillar acidic protein; HBT, Hepes-buffered Tyrode solution; Pr, propionyl moiety.

0006-291X/97 $25.00

npleteness of acylation was monitored at each stage by the stan-rd ninhydrin test (10). In cases where incomplete coupling was ınd, the coupling procedure was repeated or the acetylation was rried out before the removal of the Nα-amino protecting group ior to the coupling of the next amino acid. Acetylation was per-med with 30 % acetic anhydride in dichloromethane for 10 and minutes. Intermediate deblocking was achieved with 50% trifluo-acetic acid in dichloromethane containing 0.5% dithiothreitol fol-ved by neutralization with 10 % triethylamine in dichloromethane. ter completion of the synthesis and removal of the Nα-Boc pro-cting group from amino-terminus, antagonistic peptide was acy-ted with propionic acid (Pr) using symmetrical anhydride method. nal deprotection as well as the cleavage of the peptides from the sin were performed with anhydrous hydrogen fluoride in the pres-ıce of 8% anisol, 2% dimethylsulfide, 2% p-cresol, and 2% thiocresol 0°C for 60 min. After the removal of the hydrogen fluoride under stream of nitrogen and in vacuo, the free peptides were precipitated ith diethyl ether, filtered, washed with diethyl ether and extracted ith 50% aqueous acetic acid (short peptides) or 95% TFA (long eptides), diluted with water, and lyophilized.

Purification of peptides. Crude peptides were purified by using a imadzu LC-8A preparative HPLC system equipped with a PrepPak 00×47 mm VYDAC column packed with C18 silica gel (300 Å pore ze, 15-20 μm particle size) for short peptides and a Bakbond WP 4 column (300×47 mm, 300 Å pore size, 15-20 μm particle size) for ng peptides. The column was eluted with a solvent system con-sting of (A) 0.1% aqueous trifluoroacetic acid and (B) 0.1% trifluo-acetic acid in 80% aqueous acetonitrile in a linear gradient mode .g. 10-40% B for short peptides or 20-60% B for long peptides in 0 min , flow 80 ml/min). The eluent was monitored at 220 nm. The ractions were checked by analytical HPLC, and those with purity igher than 95% with the exception of β[1-42]amyloid were pooled nd lyophilized. The HPLC analyses of the peptides were carried out rith a Hewlett-Packard Model 1050 liquid chromatograph using a ychrosorb 5 RP-18 (250×4 mm) reversed-phase column for short eptides and a Nucleosil 300 C4 (280×4 mm) reversed-phase column or long peptides and a gradient elution with two solvent systems escribed above at a flow rate of 1 ml/min. The peaks were monitored t 220 nm. Amino acid analyses of the purified peptides were carried ut on a Hewlett-Packard Amino Quant amino acid analyzer after ydrolysis of the samples in 6 M hydrochloric acid at 110°C for 48 r in tubes sealed under vacuum. Amino acid analysis of the hydroly-ates of peptides showed the expected amino acid composition (data ot shown). Data of electrospray mass spectrometry (ES-MS, Finni-anMat TSQ 7000 mass spectrometer) were in accordance with the alculated average molecular masses.

Cell culture, treatment and fluorescence measurements. Primary ultures of rat astrocytes were prepared from the cerebral hemi-pheres of newborn rat pups by mechanical dissociation. 1 hemi-phere was dissociated in 20 ml HDMEM (DMEM with 4500 mg/l glucose) containing 10% heat inactivated fetal calf serum (Gibco) according to [11] with modifications [12-13]. Cells in HDMEM were plated onto 13×10×1 mm glass coverslips pretreated with poly-L-ornithin and fibronectin, and were placed on 12-well culture dishes and cultured in HDMEM for 4-6 weeks at 37°C in humidified 5% CO_2 atmosphere. At this stage, about 95% of the cells were GFAP positive by immuno-cytochemical staining. The cells on coverslips were exposed to 1 μmol/l final concentration of the different β-amyloid peptides for 8 hours. Intracellular loading of Fura-2-AM was attained by incubating the cells with 2 μM Fura-2-AM during the final 30 minutes of the 8-hour, 37°C incubation period. The cells on coverslips were then washed by HBT solution, pH=7.4, containing 1.8 mM $CaCl_2$ and the coverslips were transferred to cuvettes containing HBT at 25°C for measurements. Fluorescence measurements in standard 1×1 cm cross section quartz cuvettes were performed with a Hitachi F-2000 spectro-fluorimeter. Ratiometric fluorescence determinations utilised excitation at 340 and 380 nm, using emission wavelength of 495 nm at 10 nm bandwiths, the 367nm-excitation being taken as a Ca^{2+}-insensitive wavelength [14]. Total protein contents of the coverslips were determined according to [15].

TABLE I

Effect of β-Amyloid[1-42] Peptide and Its Shorter Fragments on the 340nm/380nm Fluorescence Excitation Ratios and Steady-State Fluorescence Intensities at 367 nm Excitation, Using 495-nm Emission Wavelength in Cultured Rat Astroglial Cells

Treatment	340/380 fluorescence excitation ratio	Fluorescence excited at 367 nm	Number of coverslips
Control	1.25 ± 0.06	355 ± 20	51
β[1-42]	1.39 ± 0.04	367 ± 22	18
β[1-40]	1.37 ± 0.06	360 ± 18	9
β[25-35]	1.38 ± 0.05	358 ± 16	12
β[31-35]	1.41 ± 0.04	373 ± 17	12

The cells were cultured with or without the peptides for 8 hours at 37°C and were labelled with 2 μM Fura-2-AM at 37°C for 30 minutes prior to measurements. The fluorescence excitation ratios were calculated from fluorescence intensities observed at 495nm using excitation wavelengths of 340 and 380 nm, whereas steady-state fluorescence intensities were measured at 495 nm using 367 nm excitation wavelength. The mean fluorescence intensity of unlabelled cell samples (blank) excited at 367 nm was 45 ± 11, and no difference was found between blanks taken for the control or treated cell populations. The number of coverslips investigated is also presented.

RESULTS AND DISCUSSION

Cultured rat astroglial cells were found to be labelled satisfactorily with 2μM Fura-2-AM for 30 minutes at 37°C by passive diffusion. The steady-state fluorescence intensities of unlabelled cells at 495 nm using the Ca^{2+}-insensitive excitation wavelength of 367 nm were found to be 45±11 (n=11), whereas those of labelled with Fura-2-AM ranged between 350 and 380, indicating that labelling induced a rougly 9-fold increase in the fluorescence level.

The effect of different β-amyloid peptides on the intracellular Ca^{2+} level was monitored by comparative fluorimetric studies of rat astroglial cells grown on coverslips. The cells were exposed to 1 μM final concentration of the different amyloid peptides for 8 hours. Values of the 340/380 fluorescence excitation ratio at 495-nm emission wavelength were computed after each fluorescence measurement. These results, and the steady-state fluorescence intensities using the Ca^{2+}-insensitive excitation wavelength of 367 nm, are presented in Table I. It is seen that the ratio of the 495 nm fluorescence intensities excited at 340 nm and 380 nm (340/380 fluorescence excitation ratio) was consistently higher in treated cells as compared to their untreated counterparts. Importantly, no significant alteration was detectable in Fura-2 fluorescence

TABLE II

Effect of Pr-Ile-Ile-Gly-Leu Peptide on the β[1-42]Amyloid-Induced Change in the 340nm/380nm Fluorescence Excitation Ratios and Steady-State Fluorescence Intensities at 367 nm Excitation. Using 495-nm Emission Wavelength In Cultured Rat Astroglial Cells

Treatment	340/380 fluorescence excitation ratio	Fluorescence excited at 367 nm	Number of coverslips
Control	1.22 ± 0.06	352 ± 18	16
β[1-42]	1.41 ± 0.05	359 ± 21	16
Pr-IIGL	1.26 ± 0.04	361 ± 17	16
β[1-42] + Pr-IIGL	1.28 ± 0.04	358 ± 19	16

Other conditions were as described in Table I. The number of coverslips investigated is also presented.

using the Ca^{2+}-insensitive excitation wavelength of 367 nm, indicating that the observed response reflects a real change in the Ca^{2+} concentration of the cells (Table I.).

During the 8-hour incubation period, the treated and untreated cell populations were cultured in media of identical composition except for the presence or absence of the β-amyloid peptides, the observed difference in the 340/380 fluorescence excitation ratio of Fura-2-loaded cells can be exclusively associated with the presence or absence of β-amyloid peptides, and can therefore be regarded as an effect induced by the actual β-amyloid peptide. All the peptides investigated induced similar cellular responses, suggesting that the [31-35] region of the peptide sequence could be the putative active centre of the molecule.

In order to rule out any possible artefacts caused by gross alterations in the cell number (cell mass) present actually on the coverslips, the total protein content of the coverslips was also determined. Coverslips with untreated cells were found to contain 123.1±5.7 μg protein/coverslip, whereas those of treated with β[1-42]-amyloid contained 118.6±4.7 μg protein/coverslip. This difference is not significanyt in statistical terms, so we conclude that the total protein content of treated and untreated cells did not differ considerably.

Co-treatment of the cells with equimolar concentration of Pr-Ile-Ile-Gly-Leu-NH2 peptide, an analogue of the [31-34] sequence, was found to antagonize the β[1-42]-amyloid-induced increase in the 340/380 fluorescence excitation ratio, leaving the Ca^{2+}-insensitive 367-nm fluorescence unchanged (Table II.). To the best of our knowledge, this is the first report on an analogue of β-amyloid peptide capable of blocking one of its physiological effects. This finding raises the tempting possibility that this sequence could prove to be a lead compound for designing effective β-amyloid antagonists.

It is concluded that (i) rat astroglial cells respond to treatment with these peptides with a long-term elevation of intracellular Ca^{2+}-concentration; (ii) the observed change can be considered as a long-term cell physiological action and can be used as a sensitive marker for testing for long-term activities of β-amyloid peptides; and (iii) Pr-Ile-Ile-Gly-Leu-NH2 peptide appears to be capable of preventing the β[1-42]-amyloid-induced long-term elevation in $[Ca^{2+}]i$.

ACKNOWLEDGMENTS

The research reported here was supported by the grants Nos. T013029, T017751, T022542, T022546 from the Hungarian Scientific Research Fund (OTKA) and by the grant No. T-04 522/93 from the Hungarian Medical Scientific Council (ETT). The excellent technical expertise of Marton Zoltánné and Katalin Ôri in cell culturing is appreciated.

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II.

NeuroReport 10, 1693–1698 (1999)

Propionyl-IIGL tetrapeptide antagonizes β-amyloid excitotoxicity in rat nucleus basalis

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A putative tetrapeptide β-amyloid (Aβ) antagonist (propionyl-Ile-Ile-Gly-Leu [Pr-IIGL]) based on the [31-34] sequence of Aβ was previously shown to rescue astrocytes from Aβ-induced membrane depolarization and subsequent long-term elevations of the intracellular Ca^{2+} concentration *in vitro*. Here we provide *in vivo* evidence that the Pr-IIGL tetrapeptide effectively attenuates the excitotoxic action of Aβ(1-42) on cholinergic neurons of the rat magnocellular nucleus basalis (MBN). We also demonstrate by means of microdialysis that administration of Pr-IIGL abolished Aβ(1-42)-induced increases in extracellular aspartate and glutamate concentrations in the MBN, which coincide with a significant preservation of cholinergic MBN neurons and their cortical projections. This neuroprotective effect was associated with preserved exploratory behavior in an open-field paradigm, and improved memory retention in a step-through passive avoidance task. Our data presented here indicate for the first time the efficacy of short, modified functional Aβ antagonists in ameliorating Aβ excitotoxicity *in vivo*. *NeuroReport* 10:1693–1698 © 1999 Lippincott Williams & Wilkins.

Key words: β-Amyloid; Excitotoxicity; Glutamate; Learning and memory; NMDA receptor; Tetrapeptide antagonist

Introduction

One of the major neuropathological characteristics of Alzheimer's disease is the extensive accumulation of β-amyloid peptides (Aβ) forming senile plaques in brain areas involved in information storage or processing [1]. Ample *in vitro* [2,3] and *in vivo* [4–9] experimental data indicate that Aβ fragments are toxic to neurons, in particular to cholinergic projection neurons of the rat magnocellular nucleus basalis (MBN) and medial septal complex [4–9]. In fact, an excitotoxic *in vivo* cellular pathway, involving activation of NMDA receptors and subsequent intracellular Ca^{2+} overload, has been postulated recently [6]. In this regard, pharmacological manipulation of Ca^{2+}-permeable receptor channels was reported to inhibit Aβ toxicity with high efficacy [6,7]. An alternative strategy in designing potential anti-Aβ drugs is to prevent or inhibit Aβ aggregation using synthetic Aβ derivatives, termed β-sheet breaker peptides [10]. Indeed, Aβ fibril formation and neurotoxicity was effectively attenuated by peptide fragments derived from the 16-20 region of the Aβ sequence [10–12]. Other lines of evidence indicate that synthetic Aβ-related peptides generated by extensive modification of the active core of Aβ, particularly the β(31-35) sequence, are able to attenuate the cytotoxic action of Aβ [2]. The neurotoxic cascade by which these putative Aβ antagonists protect nerve cells presumably involves selective antagonism of cellular Aβ recognition and cell-surface binding [2,13], while Aβ folding or aggregation is not influenced. In this respect, recent data in our laboratories indicate that the potential Aβ antagonist propionyl-Ile-Ile-Gly-Leu tetrapeptide (Pr-IIGL), derived from the 31-34 sequence of Aβ(1-42), prevents membrane depolarization (unpublished observations) and subsequent elevation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of cultured astrocytes, elicited by Aβ(1-42) or its fragments, Aβ(1-40), Aβ(25-35) or Aβ(31-35) [2].

In the present study the neuroprotective potential of Pr-IIGL was evaluated by means of correlative behavioral, neurochemical and anatomical ap-

proaches in an *in vivo* lesion paradigm. Effects of Pr-IIGL in diminishing Aβ(1-42) excitotoxicity were determined by means of retrograde microdialysis of Aβ(1-42) and/or Pr-IIGL with simultaneous measurement of extracellular excitatory amino acid concentrations in the MBN of freely moving rats. Subsequently, microdialyzed animals were tested in an open-field and a one-way step-through passive avoidance task to assess functional recovery following Pr-IIGL + Aβ(1-42) administration, whereas quantitative acetylcholinesterase (AChE, EC 3.1.1.7) histochemistry was employed to demonstrate the protective action of Pr-IIGL on cholinergic projections of MBN neurons 14 days post-surgery.

Materials and Methods

Peptide synthesis: Both Pr-IIGL and Aβ(1-42) were synthesized with amide at the C-terminal by a solid-phase technique involving Boc chemistry as described previously in detail [2,4,6,7]. In brief, Aβ(1-42) was synthesized on an ABI 430A automated peptide synthesizer and peptide chains were elongated on *p*-methylbenzhydrylamine (MBHA) resin (0.57 mmol/g). Couplings were performed with dicyclohexylcarbodiimide with the exception of Asn and Gln, which were incorporated with *N*-hydroxybenzotriazole ester to avoid side reactions [2]. After completion of the synthesis the antagonistic peptide was acylated with propionic acid (Pr) using a symmetrical anhydride method [2]. Final deprotection and cleavage of the peptides from the resin were carried out with hydrogen fluoride at 0°C for 60 min. Subsequently, free peptides were precipitated, solubilized, filtered and lyophilized. Crude peptides were purified by using a Shimadzu LC-8A HPLC system equipped with a PrepPak 300 × 47 mm C_{18} VYDAC (for Pr-IIGL) or a Backbond WP C_4 column (for Aβ(1-42)). In both cases, amino acid analyses demonstrated the expected amino acid composition and electrospray mass spectrometry gave the expected molecular masses.

Animals and surgical procedure: Young adult male Wistar rats (300–350 g; $n = 16$, sham-operated ($n = 6$), Aβ(1-42)-infused ($n = 6$), Pr-IIGL + Aβ(1-42)-treated ($n = 4$); Charles River, Hungary) were individually housed for at least 3 days prior to the experiments and kept on normal laboratory diet and tap water *ad lib* in an air-controlled room (21 ± 2°C) with a 12:12 h light:dark cycle (lights on at 06.00 h). The animals were anesthetized with sodium pentobarbital (30 mg/kg, i.p., Sigma) and Hypnorm (0.4 mg/kg, i.m., Janssen Pharmaceuticals, Belgium), and their heads mounted in a stereotaxic frame (Narishige, Japan). A concentric microdialysis probe, made of Travenol hollow fibers (2 mm diffusion length, 0.2 mm in diameter, 50 kDa cut off) [14] was implanted into the right MBN at standard coordinates (AP −1.5 mm, L 3.2 mm, DV 6.5 mm [15]) [4–7]. Subsequently, the microdialysis probe was secured to the skull with dental acrylic. Inlet and outlet tubes of the probe were run through a liquid swivel to allow collecting dialysis samples from freely moving conscious animals. In accordance with the European Communities Council Directive (86/609/EEC), all efforts were made to minimize animal suffering throughout the experiments.

Pr-IIGL or Aβ(1-42) microdialysis and amino acid analysis: Dialysis was started 24 h after probe implantation. Artificial cerebrospinal fluid (ACSF; 147 mM Na^+, 3.5 mM K^+, 2 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.3) served as vehicle throughout the experiments. Both Pr-IIGL and Aβ(1-42) were used in equimolar concentration (200 μM) and solubilized in ACSF to which 0.1% trifluoroacetic acid (TFA) had been added. Vehicle + TFA-dialyzed animals served as controls as the treatment protocol and pH shift of the vehicle were previously demonstrated to produce neither excitotoxic neuronal damage nor influence the extracellular amino acid concentrations in the MBN (data not shown) [16]. After a 60 min equilibration period three dialysis fractions were collected. Baseline of the dialysate was calculated as the average of the three baseline measures. Subsequently, Pr-IIGL was infused for 60 min (pretreatment) which was followed by a 40 min infusion of Aβ(1-42). A constant, 2 μl/min flow rate was used throughout. Outflow fractions were collected in 10 min blocks to determine the excitotoxic action of Aβ(1-42) and its prevention by pre-administered equimolar Pr-IIGL.

Extracellular amino acid levels were determined after precolumn derivatization by isocratic HPLC analysis of ortho-phtalaldehyde (OPA) derivatives of amino acids as described previously [17]. The derivatizing reagent was added separately to the microdialysis samples, mixed for 5 s and immediately loaded into the 50 μl sample loop of an HPLC valve and onto the column [18]. A second loop of 300 μl was continuously filled with 90% ethanol and directly after the GABA peak was recorded, the loop content was injected onto the column to quickly remove all subsequent peaks. The HPLC system consisted of an LKB 2150 HPLC pump (Pharmacia, the Netherlands), an S3 ODS2 Spherisorb column with 3 μm particle size, Rheodyne valves (CA, USA) and a fluorimeter (Shimadzu RF-10A, Sweden). Fluorimetric detection was per-

formed at 340 nm excitation and 400 nm emission wavelengths. A mobil phase consisting of 0.05 mol/l Na_2HPO_4, 0.01 mmol/l Na_2-EDTA, 0.6% (v/v) tetrahydrofuran and 30% (v/v) methanol (pH 6.8 adjusted with phosphoric acid) was used. The derivatization reagent was prepared as follows: 5 mg OPA (Sigma), was dissolved in 50 µl methanol and added to 5 ml 0.5 mol/l $NaHCO_3$ (pH adjusted to 9.5 with NaOH) containing 15 µl 2-mercaptoethanol. The reagent was prepared daily. Aspartate (Asp), glutamate (Glu) and taurine were separated appropriately. Aβ filtration through the probe was checked *in vitro* by measurement of the concentration difference between in- and outflow [^{13}H]Aβ(25-35) contents of perfusates using a liquid scintillation counter.

Behavioral tests: Novelty-induced spontaneous activities of the animals were assessed 24 h post-surgery in the open-field paradigm, as described previously [5]. Each test lasted for 3 min, during which latency to the start of exploration, the degree of horizontal ambulation, and the number and total time a rat spent in an upright position (rearing) were recorded. Animals were also tested for discriminative learning in a one-way step through passive avoidance task 14 days post-surgery [5]. In the training trial (day 12) the animals were placed in an illuminated chamber to explore the boxes. The latency to step into the dark compartment was recorded (preshock latency, data not shown). On the second day of the test (day 13) a mild foot shock (1 mA, 3 s) was delivered through the grid floor upon entry into the dark compartment. Retesting was performed 24 h later (day 14) and the latency to step into the dark chamber was recorded (post-shock latency) within a total of a 3 min retesting period.

Tissue processing and quantitative AChE histochemistry: Fixation of the brains was carried out under deep sodium pentobarbital anesthesia by transcardial perfusion with 400 ml fixative composed of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), which was preceded by a short pre-rinse with physiological saline. Brains were postfixed for 4 h in the same fixative, cryoprotected by overnight storage in 30% sucrose in 0.1 M PB at 4°C. Thereafter, coronal frozen sections were cut on a cryostat microtome at 20 µm. For AChE histochemistry, free-floating sections were postfixed by immersion in a 2.5% glutardialdehyde solution in PB saline overnight at 4°C. Cholinergic fibers were visualized by staining for AChE according to Hedreen *et al.* [19] using a silver nitrate intensification procedure.

AChE fiber density was measured in layer V of the posterior somatosensory cortex according to a standard protocol [5,7] using a Quantimet Q-600HR computerized image analysis system (Leica, Rijswijk, the Netherlands). Surface area density of cortical AChE-positive fibers (the area covered by AChE-positive cholinergic fibers/the total sampling area, given as percentages) was measured in two parietal cortical sections (at coordinates −1.3 mm and −1.7 mm [17]), representing the densest cholinergic innervation from the damaged MBN division [5,7]. After background substraction and gray-scale threshold determination, the surface area of skeletonized AChE-positive fibers was computed in each parietal cortical section using a 599 nm emission filter. The relative value of fiber reduction was calculated in pre-established quadrants as the percentage difference between the surface area density at lesioned and contralateral sides of the brain [7].

Statistical analysis: Results of extracellular amino acids (between experimental groups) as well as the loss of cortical AChE-positive fibers were statistically evaluated with either two-way or one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls *post hoc* test (SPSS for Windows, Release 6.1.2, 1995, SPSS Inc.). Time-dependent alterations of extracellular amino acids within single experimental groups were evaluated by repeated measures ANOVA (SPSS for Windows). Alterations of spontaneous behaviors in the open-field, and changes in passive avoidance learning were analyzed with the non-parametric Mann-Whitney test (MINITAB release 9.2, 1993, Minitab Inc. State College, PA, USA). $p < 0.05$ was taken as indicative of statistical significance for the tests. Data on biochemical and histochemical parameters were expressed as means ± s.e.m., while data on both open-field and passive avoidance behaviors represent medians.

Results

Infusion of Pr-IIGL in the MBN elicited a significant extracellular elevation of Asp, Glu and taurine, with postponed peak responses 40 min after the start of peptide infusion ($p < 0.05$; Fig. 1). Interestingly, following a short excitatory response, excess extracellular Asp and Glu levels returned to the control (baseline) value. In contrast, Aβ(1-42) infusion in the MBN elicited sustained elevation of excitatory amino acids (Fig. 1), which was apparently present between 10 and 60 min following dialysis of Aβ(1-42) ($p < 0.01$ *vs* baseline level). Aβ(1-42) induced extensive increases in Asp and Glu concentrations with an abrupt onset (100% (baseline) *vs* 146 ± 34% or 198 ± 28% (Asp or Glu, respectively, at 10 min), $p < 0.05$; Fig. 1). Sixty minutes after the start of Aβ(1-42) infusion in the MBN the extracellular

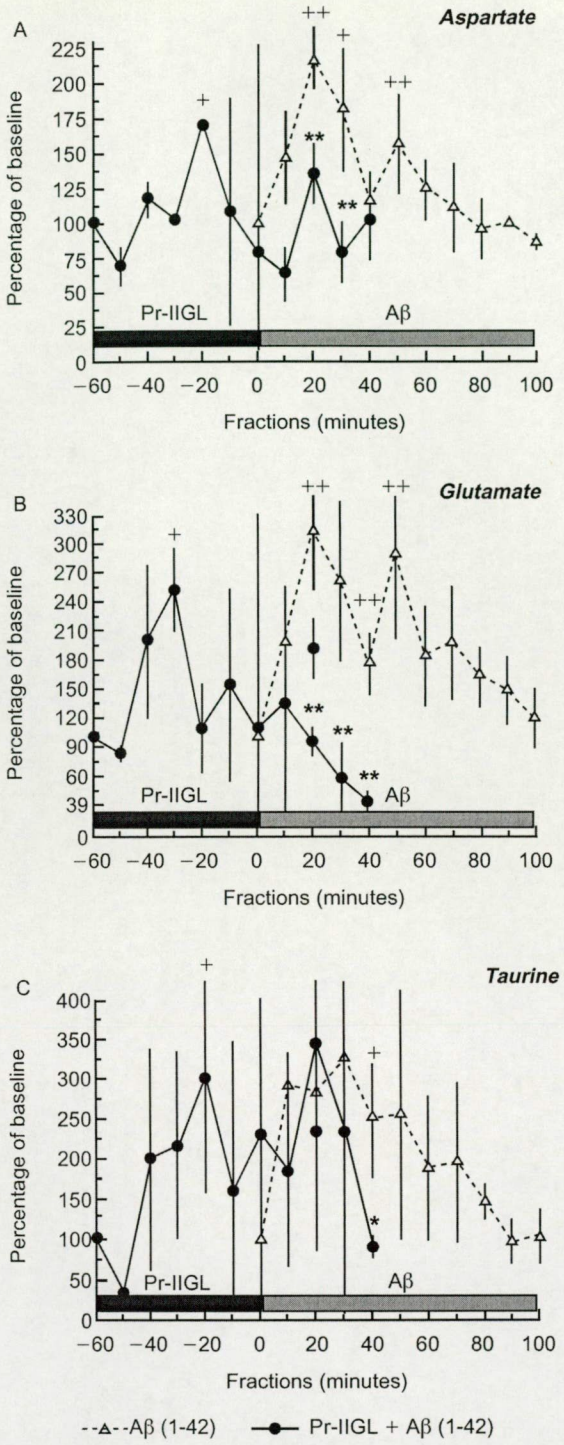

FIG. 1. Effects of Aβ(1-42) and Pr-IIGL + Aβ(1-42) on the extracellular excitatory amino acids (**A**) aspartate and (**B**) glutamate and on (**C**) taurine concentrations in the rat magnocellular nucleus basalis (MBN). Whereas Pr-IIGL transiently mimicked the excitatory action of Aβ(1-42) and thus shared agonistic properties with Aβ(1-42), it effectively decreased Aβ(1-42)-induced sustained excitation of MBN neurons. Note that Pr-IIGL did not influence the release profile of taurine, thereby enhancing the efficacy of endogeneous neuroprotection (C). ** $p < 0.01$, * $p < 0.05$ *vs* Aβ(1-42) (one-way ANOVA), ++ $p < 0.01$, + $p < 0.05$ *vs* the baseline level (100%; repeated measures ANOVA). Six Aβ(1-42)-infused and four Pr-IIGL + Aβ(1-42)-infused rats were used. Reversed treatment, e.g. Aβ(1-42) + Pr-IIGL did not result in considerable neuroprotection (data not shown).

amino acid levels gradually decreased and returned to the baseline level after 100 min. Pr-IIGL exerted a characteristic pharmacological action as it significantly decreased Aβ(1-42)-induced elevations of Asp concentration, and eventually abolished Aβ(1-42)-induced enhanced Glu release ($p < 0.01$ Pr-IIGL + Aβ(1-42) *vs* Aβ(1-42); Fig. 1). Interestingly, pretreatment with Pr-IIGL did not influence the release profile of taurine, as peak taurine concentrations after either a combined Pr-IIGL + Aβ(1-42) or single Aβ(1-42) administration did not differ significantly from each other. Reversed administration of Aβ(1-42) + Pr-IIGL did not result in any noticeable antagonistic effect (data not shown, $n = 2$).

Assessment of spontaneous behaviors 24 h postlesion revealed a pronounced increase in the open-field latencies of Aβ(1-42)-infused animals, compared with all other groups examined ($p < 0.05$ *vs* sham-operated; Fig. 2A). In parallel with the changes of start latency Aβ(1-42)-treated rats exhibited markedly decreased horizontal motor activities compared with both sham-control ($p < 0.05$; Fig. 2B) and Pr-IIGL + Aβ(1-42)-treated rats ($p = 0.071$; Fig. 2B), whereas rearing parameters did not change as a consequence of Aβ(1-42) infusion (data not shown). Aβ(1-42) microdialysis in the MBN resulted in severe disturbances of passive avoidance learning, which were indicated by significantly decreased post-shock latency periods of Aβ(1-42)-infused animals, whereas Pr-IIGL attenuated the behavioral dysfunctions (Fig. 2C).

Infusion of Aβ(1-42) in the MBN elicited a significant loss of AChE-positive projection fibers in the somatosensory cortex, compared with all other experimental groups investigated ($p < 0.01$; Fig. 3), which was effectively antagonized by Pr-IIGL pretreatment (Fig. 3).

Discussion

The present investigations point to the potent neuroprotective effects of the Aβ antagonist Pr-IIGL, which protected cholinergic MBN neurons and their cortical axonal projections against Aβ(1-42) excitotoxicity and ameliorated the behavioral effects of Aβ(1-42) infusion. Previous studies demonstrated that competitive treatment of astroglial cells with equimolar concentrations of Pr-IIGL and Aβ(1-42) results in the prevention of astroglial membrane depolarization and pathological $[Ca^{2+}]_i$ accumulation induced by the exposure of astroglial cells to Aβ(1-42) alone. With regard to the presently reported *in vivo* findings it should be noted that depolarization of astroglial membranes leads to a rapid blockade of glial glutamate uptake [20,21]. Inhibition of excitatory amino acid clearance in

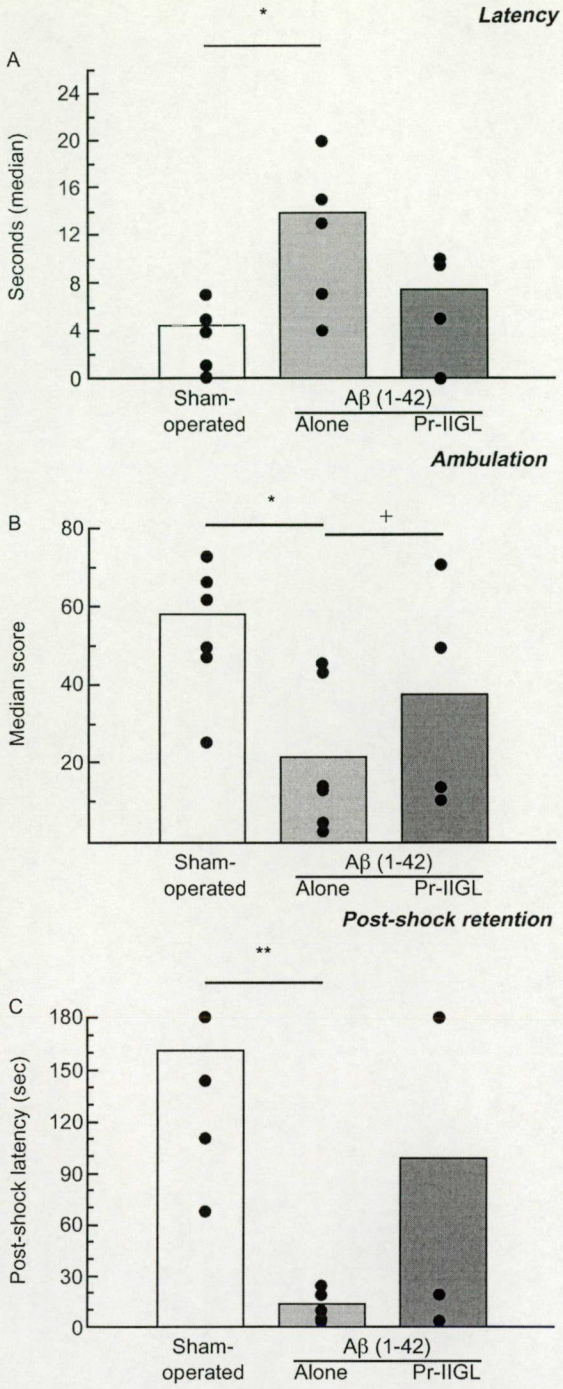

FIG. 2. The effects of Aβ(1-42) on novelty-induced behaviors in the open-field 24 h post-surgery (**A**,**B**) or short-term memory in the post-shock trial of a passive avoidance task (**C**). A significant hypoactivity of Aβ(1-42)-infused animals was recorded in the open-field arena, which was markedly attenuated by Pr-IIGL pretreatment. Moreover, prolonged Aβ(1-42) infusion into the MBN resulted in the loss of learning and memory functions, whereas Pr-IIGL partially antagonized Aβ(1-42)-induced decreases of post-shock latencies. (A) depicts the latency to start ambulation in the open-field, (B) the horizontal motor activity of the animals, while (C) shows the results of a 24 h retention trial in the passive avoidance task. ** $p<0.01$, * $p<0.05$, + $p=0.071$ Aβ(1-42) *vs* sham-operated or Pr-IIGL + Aβ(1-42) treatment (Mann-Whitney test). Six sham-operated, six Aβ(1-42)-infused and four Pr-IIGL + Aβ(1-42)-infused rats were used. Data were expressed as medians. Vertical bars correspond to the median value of each experimental group, while solid circles denote individual behavioral parameters.

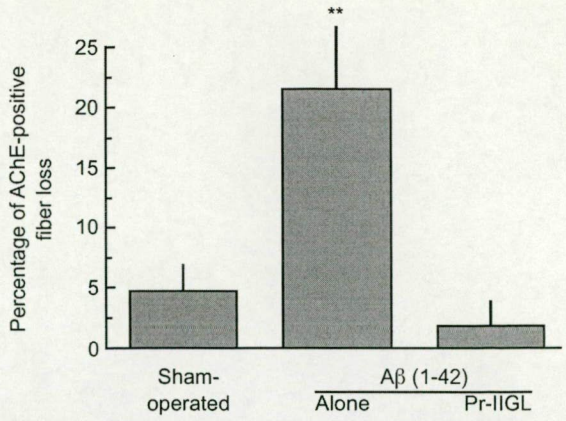

FIG. 3. Loss of cholinergic (AChE-positive) projection fibers 14 days post-surgery as a consequence of Aβ(1-42) infusion into the MBN and the neuroprotective effects of the conformational Aβ antagonist, Pr-IIGL tetrapeptide. ** $p<0.01$ Aβ(1-42) *vs* sham-operated or Pr-IIGL + Aβ(1-42) treatment (one-way ANOVA followed by Student–Newman–Keuls *post hoc* test). Six sham-operated, six Aβ(1-42)-infused and four Pr-IIGL + Aβ(1-42)-infused rats were used. Data were expressed as means ± s.e.m.

microglia might readily be implicated in the *in vivo* neurotoxic cascade of Aβ as the peptide may act as an effective trigger of the extracellular elevation of amino acids, leading to neuronal damage. Such an excitotoxic cascade of events after Aβ infusion may also be concluded from the significantly increased extracellular levels of Asp, Glu and taurine that were recorded shortly after the start of Aβ(1-42) infusion with peak concentrations of excitatory amino acids 20 min post-infusion. Moreover, Aβ(1-42) infusion in the MBN elicited widespread loss of the cholinergic projection fibers that originate from the injured MBN subdivision. Recent data suggest that the magnocellular basal forebrain is primarily involved in the subcortical regulation of neocortical and limbic arousal-associated and cognitive functions [22]. Neurotoxic lesions in the MBN therefore result in severe disturbances of the tonic regulation of the cholinergic basal forebrain with a direct impact on learning and memory retention mechanisms assigned to neocortical structures [23]. Thus, Aβ(1-42)-induced hypoactivity of the cholinergic neurotransmission in the cortical innervation areas might critically deplete spontaneous animal mobility and hamper retention mechanisms during passive avoidance learning [22,23]. Taking our data obtained by microdialysis with long-lasting behavioral deficits and the extensive loss of cholinergic projection fibers together, Aβ(1-42) compromises MBN neurons via an excitotoxic cascade, as suggested previously [3,6,24]. As *in vitro* testing of the transmembrane outflow of [^{13}H]Aβ(25-35) revealed 6.88% peptide utilization, microdialysis of Aβ(1-42) during the 100 min test sessions is equal to the effects of ~16.51 µl of 0.2 nmol/µl Aβ(1-42) acutely injected into the MBN [5,6,16].

In addition to conventional pharmacological approaches, such as selective blockade of voltage-dependent Ca^{2+} channels [7,16] or application of antioxidants [6], arrest of Aβ fibril formation [10,11], Aβ aggregation or amyloidogenicity [12] has recently become of therapeutic relevance to Alzheimer's disease. The action of Aβ-related peptide derivatives, however, might be multifold, as synthetic 16-20-derived peptide fragments, termed β-sheet breakers, were shown to hamper Aβ fibril generation [10,12], whereas a functional antagonistic role interfering with selective Aβ recognition/binding to presumed cell surface receptors was postulated for fragments derived from the 31-35 region [2]. In fact, Pr-IIGL as a representative compound of the latter family was recently shown to reduce Aβ(1-42)-induced elevation of $[Ca^{2+}]_i$ with high efficacy *in vitro* [2]. Supportive to the Aβ antagonist nature of Pr-IIGL, *in vivo* administration of the tetrapeptide exhibited slight agonistic effects resembling the excitatory action of Aβ(1-42). It is worthy of attention that Pr-IIGL abolished Aβ(1-42)-induced excess accumulation of Asp and Glu without affecting extracellular taurine levels. These data may stress the neuroprotective potential of Pr-IIGL as it selectively diminishes the release of excitotoxic amino acids without deranging the action of the endogenous neuroprotectant, taurine [25]. Moreover, preservation of cholinergic MBN neurons and their cortical projections by Pr-IIGL may account for the improvement of memory retention in the post-shock trial of the passive avoidance learning task.

Based on our current and previous data [2], it is assumed that Pr-IIGL interacts with cell surface recognition sites (receptors) for Aβ [6,13]. Thus, Pr-IIGL acts as a characteristic Aβ antagonist and may displace selective Aβ binding from its putative receptor, such as the NMDA receptor channel [13], which might, in turn, result in the prevention of unbalanced changes of $[Ca^{2+}]_i$.

Conclusion

The present *in vivo* studies provide coherent neurochemical, anatomical and behavioral experimental evidence that Aβ(1-42) induces neuronal injury by deranging both local homeostasis-stabilizing glial functions and nerve cells via an excitotoxic cascade involving excess extracellular accumulation of Asp and Glu, activation of voltage-dependent receptors and a subsequent Ca^{2+} overload. The present *in vivo* data indicate for the first time that a pathological enhancement of excitatory input to cholinergic MBN neurons might be of critical importance in Aβ(1-42)-induced neuronal damage. Moreover, Pr-IIGL, a putative Aβ tetrapeptide antagonist, obtained by synthetic modification of the β(31-34) region, prevents the excitatory action of Aβ(1-42) in the rat MBN. Hence, Pr-IIGL protects cholinergic MBN neurons against Aβ(1-42)-induced neuronal damage which results in functional recovery of the animals. The underlying mechanism of the neuroprotective action of Pr-IIGL might entail ligand-like displacement of Aβ(1-42) binding from its hypothetical receptor.

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ACKNOWLEDGEMENTS: The authors thank Dr Gábor Juhász and Dr Mária Sasvári for their expert help during the initial phase of microdialysis experiments. This work was supported by OTKA, Hungary (F23865 to T.H., F016560 to. I.Á., T017751 to P.B., T022546 to M.Z., T026451 to C.N., T022542 to L.G.) and a joint grant of NWO and OTKA (NWO 048-11-006 to P.G.M. L./OTKA N26674 to C.N.).

Received 24 February 1999; accepted 13 April 1999

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Journal of Molecular Structure 480–481 (1999) 481–487

Journal of
MOLECULAR
STRUCTURE

Solvent effect on aggregational properties of β-amyloid polypeptides studied by FT–IR spectroscopy

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Received 24 August 1998; accepted 4 November 1998

Abstract

Aggregation of the β-amyloid peptides is the major hallmark of the brain in case of Alzheimer's disease. On the basis of some results it is assumed that the toxic centrum of the βA4 (1–42) amyloid peptide is primarily the (31–35) fragment [N.W. Kowall, A.C. McKee, B.A. Yanker, M.P. Beal, Neurobiol. Aging 13 537–542; B. Penke, L. Tóth, K. Soós, J. Varga, E.Z. Szabó, J. Márki-Zay, A. Baranyi, in: H.L.S. Maia (Ed.), Peptides 1994, Proceedings of the 23rd European Peptide Symposium Escom, Leiden, 1995, pp. 101–102; I. Laczkó, Z. Kónya, J. Varga, K. Soós, M. Hollósi, B. Penke, in: H.L.S. Maia (Ed.), Peptides 1994, Proceedings of the 23rd European Peptide Symposium Escom, Leiden, 1995, pp. 549–550]. Two analogues of βA4 (1–42) were synthetized: one of them includes the toxic fragment (31–35) unchanged and consists mainly of hydrophilic residues, denoted as MOD-3. The other one does not contain the toxic fragment and has mainly hydrophobic residues, denoted as MOD-4. Peptides were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to have deaggregated samples. After the addition of the D_2O as second solvent, the aggregation was followed by FT–IR spectroscopy. Changes of the spectra as a function of the composition of the solvent mixtures will be shown and discussed. Based on the results, FT–IR spectroscopy seems to be a suitable analytical control in standardizing the aggregation grade of β-amyloid peptides.

Keywords: Alzheimer's disease; β-amyloids; Aggregation; Solvent effect; 2D FT–IR

1. Introduction

The amyloid precursor protein (APP) which is a neuroprotective membrane protein has a key role in Alzheimer's disease [1–5]. It has several different molecular forms; among them APP695 is particularly abundant in neurons. In addition to the main routes, the cleavage of APP could take place on alternative pathways too, producing more or less water-soluble β-amyloid polypeptides. The abnormal accumulation of these latter sequences give possibilities to form β-conformation and β-turn containing structures which leads to aggregation and water insolubility. The ensuing formation of filaments and plaques followed by a serious neuronal cell loss is characterized as the Alzheimer's disease [4–6].

Aggregation of the β-amyloids is strongly affected by concentration, pH, temperature etc. Study of the influence of the different parameters on the aggregational properties of β-amyloids and their synthetic analogues can reveal essential data to the prevention of the undesirable transformations. Standardization of the aggregation grade of β-amyloid peptides is also essential for reproducible biological experiments.

The aim of our work was to follow the aggregation of βA4 (1–42) peptide and its two synthetic

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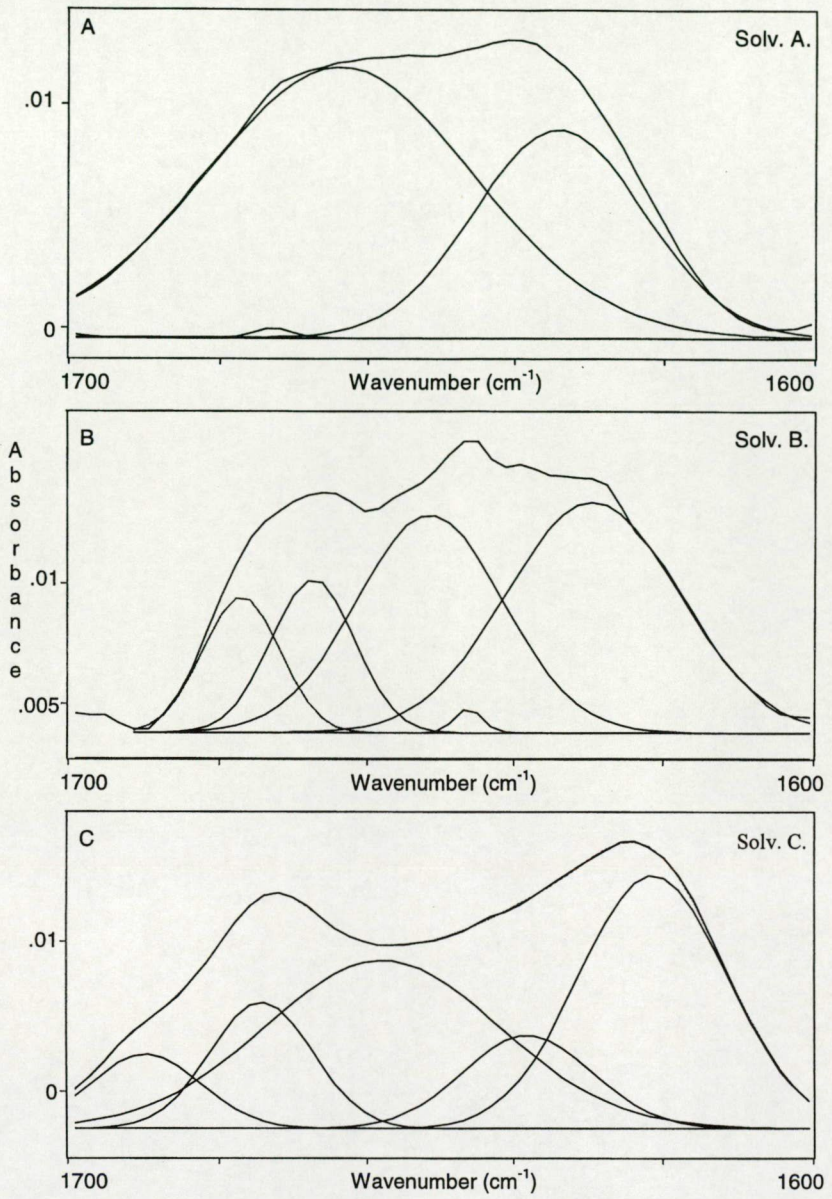

Fig. 1. Amide-I region and the curve fitting of the βA4 (1–42) peptide dissolved in (a) 90% HFIP + 10% D_2O (solv.A), (b) 50% HFIP + 50% D_2O (solv.B), (c) 10% HFIP + 90% D_2O (solv.C).

analogues: MOD-3 which was composed of the (31–35) toxic fragment and hydrophilic residues, and MOD-4 which consist of mostly hydrophobic residues without the toxic pentapeptide. In this article some results of a FT–IR spectroscopy studies including two-dimensional correlation spectra will be presented.

2. Experimental

The βA4 (1–42) peptide, MOD-3, and MOD-4 were prepared by solid phase methodology using Boc-amino acids, and were purified by reversed-phase HPLC on a semipreparative KNAUER HPLC

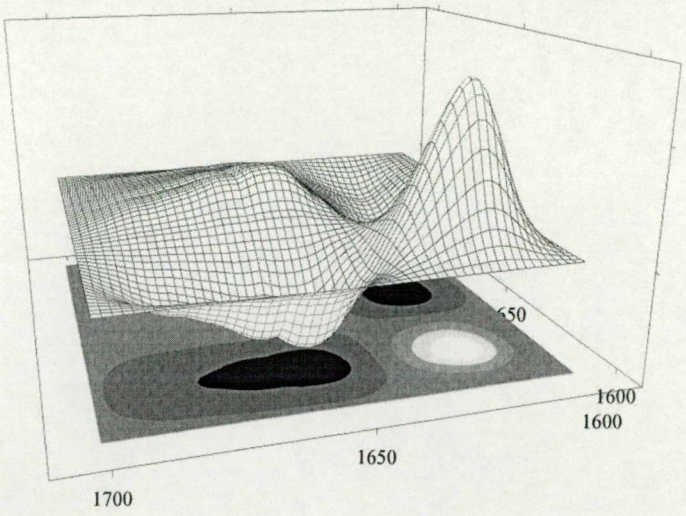

Fig. 2. Synchronous 2D IR correlation spectrum of the βA4 (1–42) peptide dissolved in 90% HFIP + 10% D_2O (solv.A).

system using Astec-300 5 C_4, BST SI-100-S 10C_{18} and Knauer Lichrosorb RP18 columns, respectively. The samples were identified by electrospray ionisation mass spectrometry (Finnigan MAT TSQ 7000). βA4 (1–42) was present as trifluoroacetic acid (TFA) salt because of the buffer system used in HPLC meanwhile MOD-3, and MOD-4 were present as acetic acid salts.

For IR measurements, the peptides were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in 10 mg/ml concentration to have deaggregated solution of the sample. As the aggregation in water is relatively fast as compared to the spectra accumulation, a PBSA/D_2O (PBSA: phosphate buffered saline) solution as buffer and second solvent was used to set different compositions of solvent mixtures, the final 1 mg/ml concentration of the peptide, and the pH = 7.4 value. Further on, those spectra will be shown and discussed which were recorded with the handling of the 90% HFIP + 10% D_2O as solv.A, 50% HFIP + 50% D_2O as solv.B and 10% HFIP + 90% D_2O as solv.C.

Spectra were recorded on a Nicolet Impact 410 instrument equipped with a DTGS detector at 4 cm^{-1} resolution. The 16 scan sample and the 16 scan background data were alternately collected for two hours or more. Spectra evaluation were carried out by the WIN-IR software package. The examined region was 1700–1600 cm^{-1}. The curve fitting was applied on the spectra at the same time elapsed.

3. Results and discussion

Detailed study of the amide-I band characteristic for peptides revealed that the position of the band depends on the secondary structures of the peptides taken part [7,8]. As these bands strongly overlap the curve fitting or deconvolution, the two dimensional spectroscopy could provide a better insight into the spectrally non-resolvable region.

The HFIP solvent is able to solve and keep the above mentioned peptides in monomeric forms which can be α-helix or random coil structure or both. In the case of the βA4 (1–42) peptide, a single but broad band mainly because of α-helix and probably solvated carbonyl groups appears between 1630–1700 cm^{-1} with a maximum at 1667 cm^{-1}. After addition of the second solvent, the aggregation starts with different rates depending on the composition of the solvent mixtures. The band characteristic for the monomeric and aggregated structures was separated by curve fitting of the amide-I region.

The spectrum of the βA4 (1–42) peptide dissolved in solv.A (Fig. 1a) shows a band at 1665 cm^{-1} similar to that of observed in pure HFIP. As the aggregation had been started, it was observed another band at

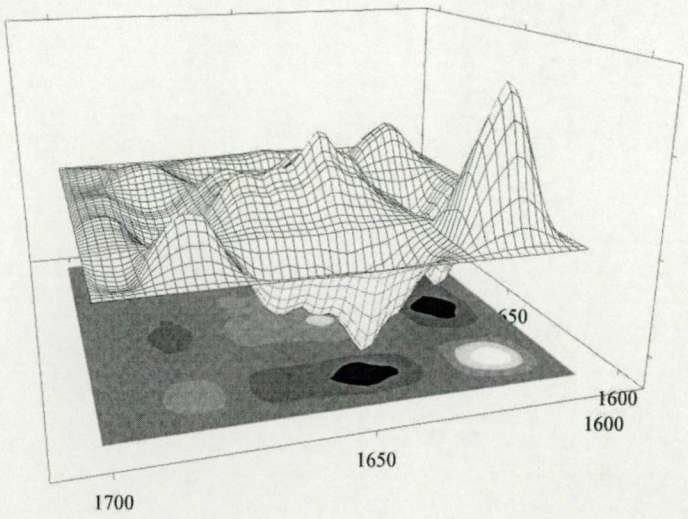

Fig. 3. Synchronous 2D IR correlation spectrum of the βA4 (1–42) peptide dissolved in 10% HFIP + 90% D_2O (solv.C).

1634 cm^{-1} with relatively medium intensity caused by β-sheet conformation. When solv.B was applied, the latter shifted to lower frequency (1630 cm^{-1}) and the broad band was separated into two narrow ones at 1667 and 1651 cm^{-1} assumed as solvated carbonyl and α-helix, respectively (Fig. 1b). In the case of solv.C, the broad band arose again with a maximum at 1657 cm^{-1}. One new band at 1638 cm^{-1} shows random coil structure. Another one at 1689 cm^{-1} with small intensity might be the high-frequency band of the antiparallel β-sheet coupled bands [8,9]. The low-frequency band of this pair appeared at 1621 cm^{-1} with quite sharper shape than in solv.B (Fig. 1c). In Fig. 1b and c the band around 1675 cm^{-1} shows a small amount of trifluoroacetic acid salt of the peptide.

In solv.A where the aggregation is relatively slow, the correlation intensity [10,11] of the β-sheet form at 1622 cm^{-1} changed into positive direction meanwhile the peak centered at 1660 cm^{-1} changed into negative direction (Fig. 2). In solv.C the β-sheet structure seems to differ from the previous one as a result of the aggregation. It is indicated in the correlation spectrum by a relatively sharp and low frequency peak at 1614 cm^{-1} that a new one appeared around 1680 cm^{-1}. The assumption that these two peaks or the corresponding bands in Fig. 1c are coupled pairs because of antiparallel β-sheet structures is supported by the fact that both have positive changes in the absorbance difference spectrum (Fig. 3).

The MOD-3 analogue was designed and synthetized in such sequence which contains the assumed toxic pentapeptide fragment at (31–35) of the βA4 (1–42) peptide and the others are mainly hydrophilic residues. The IR spectra of the samples in the different solvent mixtures used in this study gave very similar pattern to each other. For example, the spectrum recorded using solv.C (90% D_2O content) shows practically two broad bands at 1668 and 1643 cm^{-1} which arose probably from solvated carbonyl groups and monomeric forms (α-helix, random coil), respectively. This result supports our expectation: the MOD-3 peptide is not able to aggregate at all or it aggregates extremely slowly.

The MOD-4 peptide has the same amino acid sequence as βA4 (1–42) with the exception of the (31–35) fragment. The amino acids of the (31–35) fragment had been replaced by Ala with the exception of Gly. The sample dissolved in solv.A essentially consists α-helix (1658 cm^{-1}) and some random coil structure (1647 cm^{-1}). Small amount of β-sheet also appears at 1633 cm^{-1} and probably the coupled bands are shown around 1613 cm^{-1} and above 1690 cm^{-1} with week intensities (Fig. 4a). Spectrum recorded in solv.B differs basically from that of received in solv.A (Fig. 4b). The intensity of the α-helix band decreased (1657 cm^{-1}) essentially and a new band

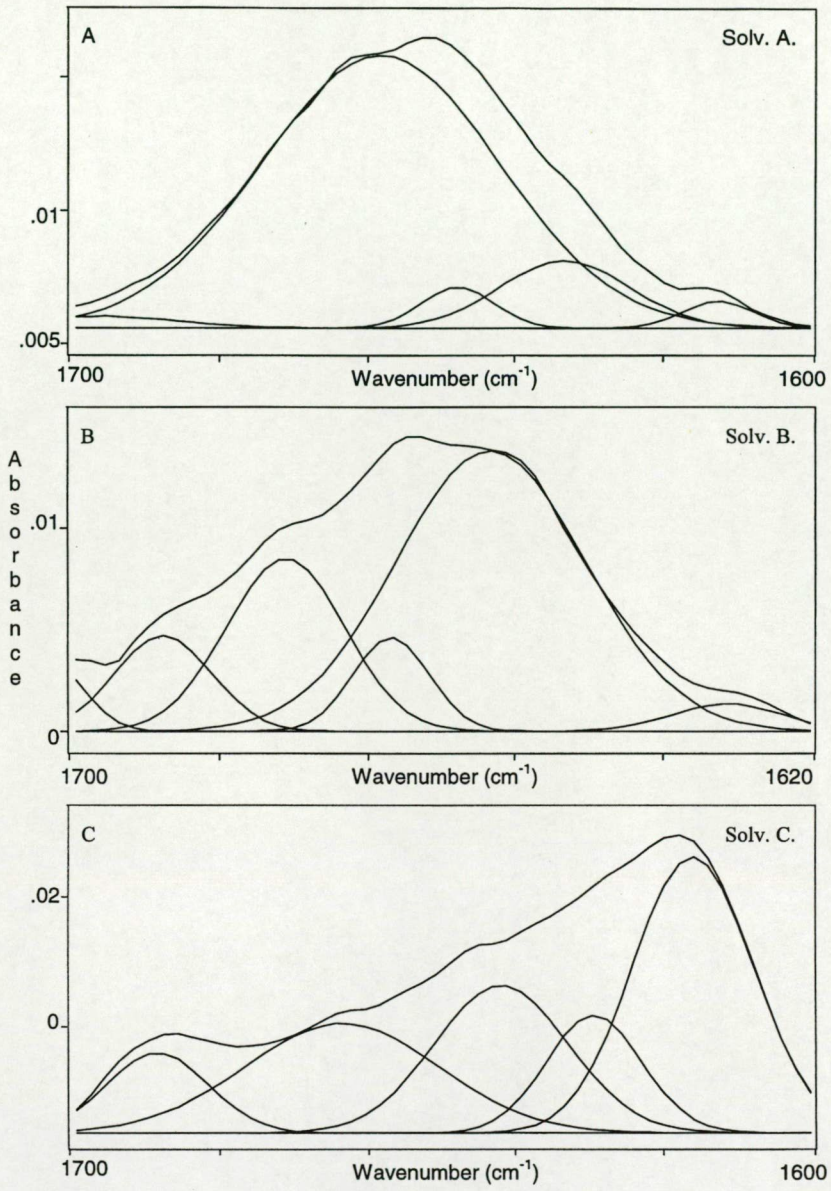

Fig. 4. Amide-I region and the curve fitting of the MOD-4 peptide dissolved in (a) 90% HFIP + 10% D_2O (solv.A), (b) 50% HFIP + 50% D_2O (solv.B), (c) 10% HFIP + 90% D_2O (solv.C).

appeared at 1643 cm^{-1} with high intensity. This band is perhaps originated from β-sheet and some random coil structure. The band at 1611 cm^{-1} have about the same intensity as in solv.A, but the high-frequency and low-intensity band of the coupled pair is depressed by a new band at 1687 cm^{-1}. The origin of that band is not exactly clear, it is assumed that it might belong to a β-turn structure. Solution of MOD-4 dissolved in solv.C shows the spectrum with the β-sheet band at 1629 cm^{-1} and the coupled bands appeared with relatively high intensity at 1616 and 1688 cm^{-1}. The latter two bands denoted antiparallel

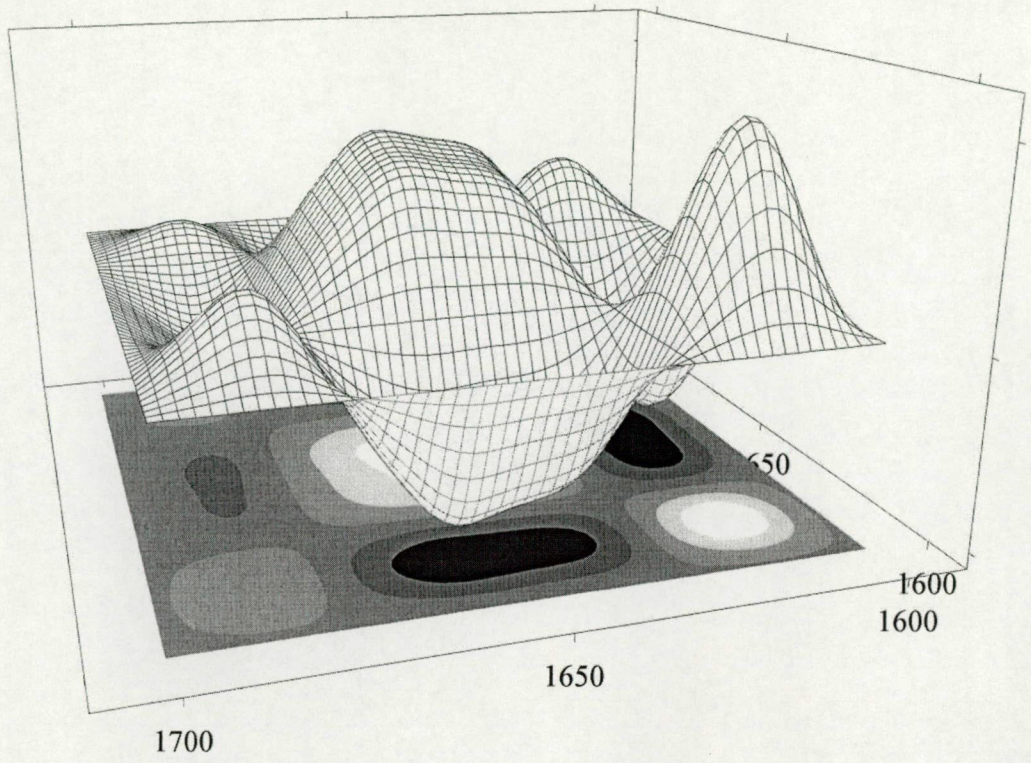

Fig. 5. Synchronous 2D IR correlation spectrum of the βA4 (1–42) peptide dissolved in 10% HFIP + 90% D_2O (solv.C).

β-sheet structure (Fig. 4c). The band from α-helix became broader with the maximum at 1663 cm^{-1} while the intensity of the random coil structure band decreased and appeared at 1642 cm^{-1}.

The 2D spectrum of the MOD-4 peptide recorded in solv.C clearly shows that the correlation intensity of the α-helix and random structures turn into well-ordered β-sheet form which is evidenced by the positively changed absorbance difference of the coupled peaks (Fig. 5).

4. Conclusions

The aggregation ability of the βA4 (1–42) and MOD-4 peptides are similar, however MOD-4 shows a small amount of antiparallel β-sheet formation even at low D_2O content. MOD-3 peptide, which has mostly hydrophilic amino acids in the sequence, shows a minimal tendency for aggregation.

Our results prove that FT–IR spectroscopy is a suitable analytical control in order to standardize the aggregation grade of β-amyloid peptides.

Acknowledgements

This work was supported by grants from the Hungarian Research Foundation (OTKA 17751, and OTKA 22546).

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Biochemical and Biophysical Research Communications 265, 297–300 (1999)
Article ID bbrc.1999.1667, available online at http://www.idealibrary.com on IDEAL®

An FT-IR Study of the β-Amyloid Conformation: Standardization of Aggregation Grade

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Received September 17, 1999

The aggregation of β-amyloid peptides is very important for their neurotoxic effect; standardization of the aggregation grade is necessary for biological experiments. Measurement of aggregation with physicochemical methods is a difficult task. The present work revealed that FT-IR can be used for studying the aggregation properties of β-amyloid peptides and the effects of environmental variables (solvent, pH, ions, and temperature) on aggregation. In dimethyl sulfoxide or hexafluoroisopropanol, amyloid peptides are in a monomeric state; on dilution with phosphate buffer just before measurement is made, aggregation begins. A detailed two-dimensional FT-IR correlation spectroscopic study was made of the conformational transitions that occur during the aggregation of β-amyloid peptides. Two processes (random/helix-to-β-sheet and aggregation of β-sheets) and multiple conformational states were observed before the most stable form was attained. β-Amyloid peptides undergo decomposition in basic buffers containing Ca^{2+}; this process should be avoided during aging experiments. © 1999 Academic Press

Deposition of the 39- to 42-amino-acid β-amyloid peptide (βA)in the brain of Alzheimer's disease patients is the most characteristic feature of this dementia of the central nervous system (1). Although the roles of β-amyloid and its aggregation into senile plaques are not yet clear, its neurotoxic effect and a correlation between the aggregation state and neurotoxic activity have been described (2). Investigations are hampered by the facts that amyloid peptides are weakly soluble under physiological conditions, and their conformation in solution is highly dependent on the solvent, the pH, the ionic strength and a number of other parameters. In the fibrillar aggregates, β-amyloid adopts an anti-parallel β-sheet conformation. Synthetic peptides from different laboratories, or even different batches from the same laboratory may exhibit significant differences in neurotoxic activity (3); these facts have been attributed to variability in the aggregation state and in the composition of different conformational states. Therefore it is important to apply some standardization method which eliminates the effects that arise from the handling of samples prior to measurement and that make the results of comparative experiments inconsistent.

To determine what parameters influence the aggregation, investigators have to work with standardized samples. This is especially true for sequence modifications, when it is not possible to prepare the standard for comparison from the same peptide sample, in contrast with studies of conformational changes caused by the solvent, different other compounds, or physical parameters. The simplest way to achieve disaggregation and a unique conformational state is dissolution in a solvent which disrupts intermolecular H-bonds (e.g., in halogenated alcohols) or all H-bonds (e.g., in dimethyl sulfoxide; DMSO), or application of a pH at which no aggregation occurs. In this work, we utilized the former method, which allows peptides to be stored deep-frozen in a monomeric stock solution until measurement.

MATERIALS AND METHODS

Aβ(1–42) and Aβ(1–40) were prepared by solid-phase methodology involving the use of Boc-amino acids. The samples were purified via a semipreparative Knauer HPLC system equipped with a reversed-phase column. The peptides were identified by electrospray mass spectrometry (ES-MS, Finnigan MAT TSQ 7000 spectrometer). The syntheses of the peptides are described elsewhere (4).

IR spectra were collected on a Nicolet Impact 410 spectrometer at room temperature in a CaF_2 cell with a 50-μm Teflon spacer. Peptide stock solutions were prepared in DMSO or hexafluoroisopropanol (HFIP) at a concentration of 10 mg/ml; they were then vortexed and sonicated for 2 min, left at room temperature overnight, and then, frozen until measurements. Stock solutions were diluted with phosphate-buffered heavy water saline (PBSA) and with additional DMSO when needed, to afford a final peptide concentration of 1 mg/ml. The spectrum of the solvent system, obtained under identical

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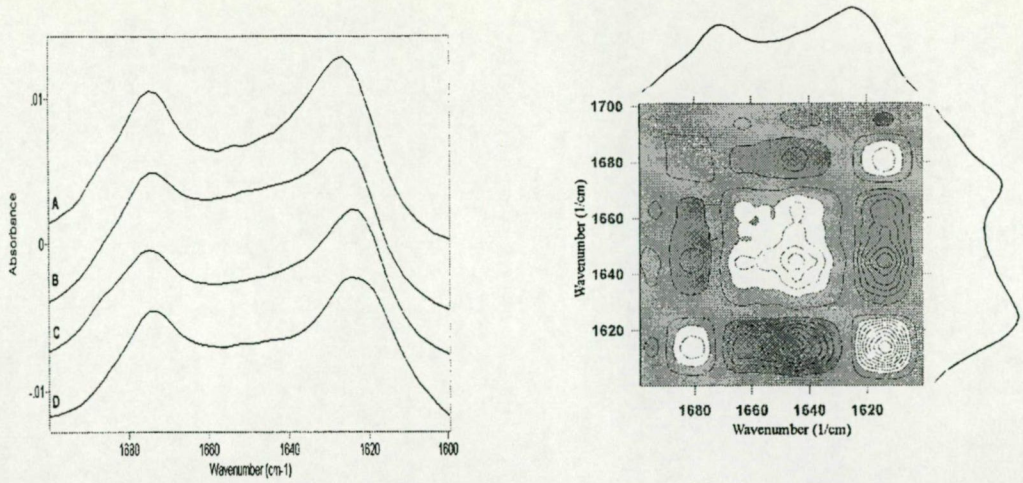

FIG. 1. (Left-hand panel) FT-IR spectra of βA(1–42) (A) and βA(1–40) at room temperature 1 min (B) or 24 h (C) after dilution with PBSA and after 60 min at 90°C in PBSA (D). (Right-hand panel) Synchronous 2D-FTIR plot of βA(1–42) in the 60-min time domain at room temperature. Average of original spectra shown above and on the right of the plots. Positive correlation intensities are white; negative correlation intensities are shaded dark.

conditions, was subtracted from the sample spectra. Most peptide samples were trifluoroacetate salts, and a spectral contribution from this counter-ion was observed at about 1675 cm^{-1}. The sample chamber of the spectrometer was continuously purged with dry N_2 and a new background spectrum was collected after 16 sample scans, without opening the spectrometer with the help of a sample shuttle. The spectral contribution of residual water vapor was eliminated by subtraction. In experiments at room temperature, a series of 128 spectra were collected, each involving 16 scans, immediately after dilution with PBSA. In experiments at elevated temperature, samples were frozen in liquid N_2 after incubating at 90°C for a given period, and 128 sample scans were collected at room temperature.

Two-dimensional FT-IR correlation (2D FT-IR) analysis was performed with a self-written program based on the algorithm of Ishao Noda (5, 6). 2D FT-IR analysis was performed in the range 1600–1700 cm^{-1} after linear baseline correction.

RESULTS AND DISCUSSION

The aggregation of amyloid peptides was studied under different conditions. To determine the effects of the conditions that were varied in the experiments, it was necessary to apply a standardization method, as amyloid peptides can exist in different conformation and aggregation states depending on the circumstances of preparation and purification; these circumstances can not be controlled, and result in batch-to-batch differences in conformation (3). Peptides were dissolved in DMSO or HFIP, and stored frozen in these stock solutions. Such stock solutions can be chosen as a unique reference start-point for aggregation and conformational studies, as amyloid peptides have been found to be monomeric (3), with a solvated unordered (in DMSO) or α-helical (in HFIP) structure (7). Dilution with PBSA at the required pH was used instead of lyophilization and redissolution because of the uncertainties in the lyophilization parameters, and since this is an easy and reliable way to initiate the aggregation. After dilution, the collection of IR spectra can be started immediately. The presence of DMSO or HFIP in the solution may alter the conformation and aggregation kinetics of the peptides, but it does not affect the results of comparative measurements. DMSO seems more suitable for such standardization: it is widely used in biological experiments, and its solutions are easier to handle, as DMSO is not as volatile as HFIP. We have studied the effects of a number of parameters on both the overall aggregation state and the aggregation process (conformational transitions during aggregation).

Conformation of βA(1–42). βA(1–42) in DMSO at a concentration of 10 mg/ml is in a solvated unordered or weakly H-bonded α-helical form, presumably in a monomer state, as indicated by other methods (3). No β-sheet bands were found (data not shown). Upon the addition of PBSA (pD 7.4) to the DMSO solution, the initial step, a conformational transition of the solvated form, began immediately, resulting in β-sheets which became more associated as time passed. The result of this process is seen in Fig. 1. The intensity of the β-sheet bands at around 1620 cm^{-1} increases as the intensity of the helical and random conformations decreases. In the second step, the position of the β-sheet peak shifts from 1623 to 1617 cm^{-1}, showing how the sheets become more extended while the interaction between them grows stronger (spectrum not shown). In the 2D FT-IR spectra (Fig. 1, left-hand), a band was found at 1689 cm^{-1}, which is positively correlated with the above β-sheet band; it can therefore be assigned as the high-wavenumber component of the antiparallel β-sheet band.

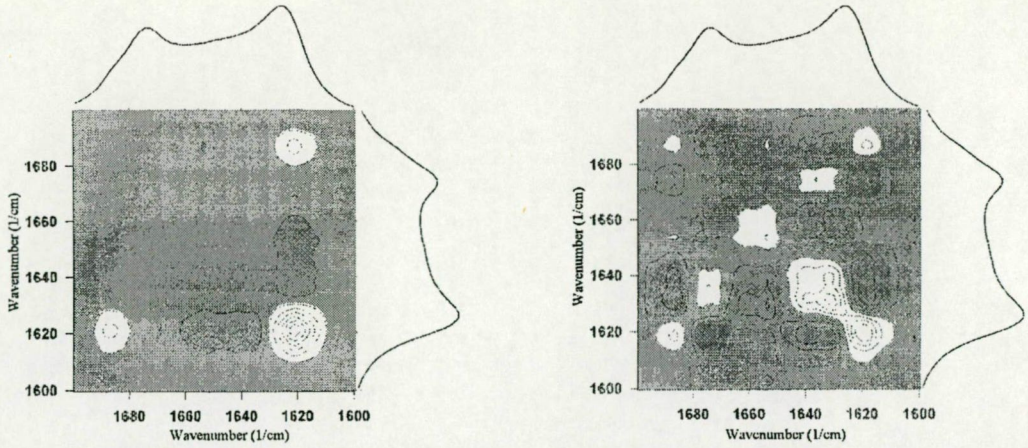

FIG. 2. Synchronous 2D-FTIR plot of βA(1–40) in the 60 min (left-hand panel) and 24 h (right-hand panel) time domains at room temperature.

Conformation of βA(1–40). βA(1–40) exhibits properties similar to those of βA(1–42), but with a smaller amount of less associated (peak at 1625 cm^{-1}) β-sheets. The difference is better visualized in the 2D FT-IR plots (Fig. 2). This is in a good agreement with the finding that this shorter analogue displays less tendency to aggregate, demonstrating the importance of the C-terminal region in the aggregation. The 2D FT-IR analysis on the spectra obtained within 1 h reveals the same random-to-sheet transformation as in the case of βA(1–42), but with a higher wavenumber for the β-sheet band. The second step of aggregation is seen from the right-hand panel of Fig. 2, with the 2D FT-IR analysis in the 24 h time domain. This shows simultaneous helix-to-sheet (1656 cm^{-1} to 1634–1678 cm^{-1} pair) and sheet-to-sheet (1634–1678 cm^{-1} pair to 1619–1685 cm^{-1} pair) transitions. From this, it can be deduced that the conversion of helical segments to β-sheets is slower than that of the random parts, which may be explained by the intramolecular H-bonds of the α-helices. The change in the β-sheet bands is a consequence of the larger splitting of the antiparallel β-sheet bands due to the association of the sheets. The former process, together with the faster random-to-sheet transition, can be attributed to nucleus formation, while the latter observations may reflect the fibril growth.

Studies at higher temperature. Samples heated at 90°C for 5, 10, 15, 30, or 60 min showed little random-to-sheet transition at this temperature, but shifts in both β-sheet bands were observed (Fig. 3). These spectral changes indicate the formation of β-sheets with stronger H-bonds (8). A highly aggregated state of the β-sheets was reached within 15 min, after which no further change could be detected at 90°C. Samples incubated at 37°C after the heating experiment showed the same random-to-sheet process as that for samples that had not been heated. These observations led to the conclusion, that βA(1–40) forms β-sheets which are stable even at high temperature and these very stable sheets are formed from intermediate β-sheets. However, formation of these intermediate β-sheets is not favored at 90°C.

Degradation of β-amyloids during aging. The amyloid peptides exert neurotoxic effects in aggregated form; solutions of these peptides were therefore used after a preaging (aggregation) procedure (several hours to several days (9, 10)). We set out to investigate the stability of the amyloid peptides under the different conditions used in most laboratories. βA(25–35), βA(1–40) and βA(1–42) were dissolved in distilled water or in buffer and their stability was checked by HPLC after several hours or days. The main peaks in the chromatograms were identified by mass spectrometry. The results were as follows:

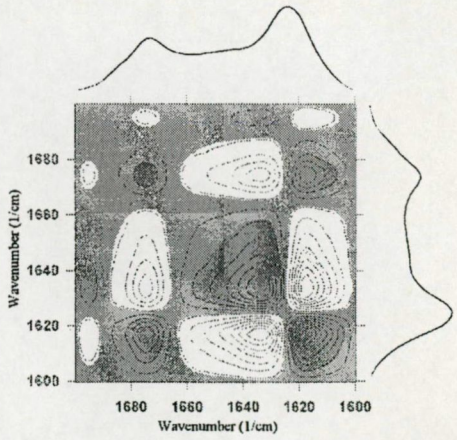

FIG. 3. Synchronous 2D-FTIR plot of βA(1–40) in the 60-min time domain at 90°C

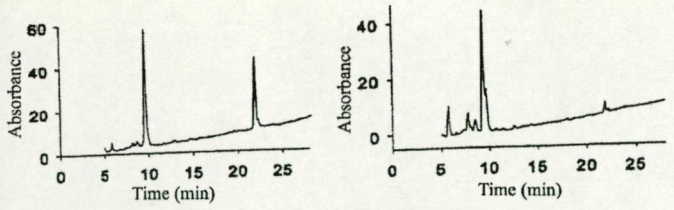

FIG. 4. HPLC of βA(25–35) after incubation for 66 h in a pH 8.0 buffer in the absence of Ca^{2+} (left-hand panel) and presence (right-hand panel) of 2 mM Ca^{2+}.

1. In distilled water at 37°C, all the amyloids were stable without any sign of decomposition even after aging for 3 days.

2. At pH 7.4 or 8.0, βA(25–35) underwent a relatively rapid decomposition if the buffer contained Ca^{2+} (Fig. 4). The main cleavage products were revealed by the mass spectra to be the tripeptide 25–27 and octapeptide 28–35. The presumed mechanism is chain cleavage via succinimide formation of Asn^{27}, which is well known in the literature (11). In pH 8.0 buffer without Ca^{2+} at 37°C, βA(25–35) underwent approximately 60% cleavage in 3 days, but in the presence of 2 mM Ca^{2+} over 95% cleavage occurred.

Preaging might be a necessary first step for amyloid aggregation before *in vivo* experiments, but it should be performed if possible at pH 7.0 in the absence Ca^{2+} and within a short time. We consider that βA(1–42) requires at most a 24-h preaging period for the formation of highly ordered aggregates with a neurotoxic effect.

In the present work, a pre-dissolution method was used for the standardization of β-amyloid aggregation. The instability of β-amyloid peptides was observed under regular conditions of aging. Nucleation and fibril growth could be followed by FT-IR spectroscopy, which allows independent studies of the effect of various compounds on either process (12–15).

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Neurobiology; 7 (4) pp. 431–436 (1999)

RESEARCH REPORT

β-AMYLOID[1-40]-INDUCED EARLY HYPERPOLARIZATION IN M26-1F CELLS, AN IMMORTALIZED RAT STRIATAL CELL LINE

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Summary: The short-term (20-minute) action of β[1-40]-amyloid on the resting transmembrane potential was investigated by means of flow-cytofluorimetric studies in M26-1F cells, an immortalized rat striatal cell line, using the potential-sensitive fluorescent probe bis-oxonol. The distribution of the individual cell-associated probe fluorescence was found to be shifted to lower levels in cells treated with β-amyloid[1-40] for 20 minutes as compared with that of their untreated counterparts. A change in the same direction was caused by valinomycin, a hyperpolarizing ionophore, whereas gramicidin, a depolarizing ionophore, induced a shift to higher fluorescence intensities. These findings, together with the reported behaviour of this particular fluorescent probe at different transmembrane potential levels, indicate that β-amyloid[1-40] is capable of inducing early hyperpolarization in M26-1F cells. This is one of the earliest cell physiological effect of β-amyloid peptides that has been reported so far. Moreover, our findings indicate an ionophore-like action of amyloid peptides.

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1218-8068/99 $ 5.00

INTRODUCTION

In recent years, considerable effort has been devoted to the elucidation of the cell physiological actions of β-amyloid peptides in neural cells. These peptides have been found to accumulate in the brain of Alzheimer's disease (AD) patients, and a causal relationship between the accumulation of ß-amyloid peptides and the onset of AD has therefore been suggested (Sisodia et al., 1990). β-Amyloid peptides are produced from the ubiquitous membrane-protein amyloid precursor protein (APP) by a pathogenic cleavage process (Haass et al., 1992) in the brain. These peptides have been shown to induce cytotoxic action in neural and glial cells, leading to cell degeneration both *in vitro* (Korotzer et al., 1993) and *in vivo* (Kowall et al., 1991). As concerns their primary cellular effects, the enhancement of protein phosphorylation (Luo et al., 1995), the modification of cytosolic (Ca^{2+} homeostasis (Mattson et al., 1993), and the production of O_2 free radicals (Schubert et al., 1995) have been reported in neural cells. Although all of these observed physiological alterations have been implicated in the development of cytotoxic symptoms in neural cells, very little is known about the connection between the early effects and the onset of cellular degeneration. The present communication reports on an early hyperpolarization of the transmembrane potential by β-amyloid[1-40] in a rat striatal cell line, suggesting that the peptide induces a rapid change in the intracellular ionic homeostasis and/or membrane permeability. Immortalized rat striatal cells (cell line M26-1F) were used in these investigations. This cell line was formerly established from embryonic rat striatal cells by using the A58 temperature-sensitive allele of the Sv40 large T antigen (Giordano et al., 1993).

MATERIALS AND METHODS

Cell culture, amyloid treatment and dye loading. M26-1F cells were cultured as described previously (Giordano et al., 1993). Briefly, the cells were cultured in poly-L-lysine-coated plastic Petri dishes by using DMEM/Nutrient Mix F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, adjusted to pH 7.4 with 1.125 g/l $NaHCO_3$, in an atmosphere of 95% O_2 and 5% CO_2 at 33 °C for 3 days. The cells were treated with 0.5% tripsin for 3 minutes and transferred to

phosphate-buffered saline solution (pH 7.4). The cells in suspension were then exposed to a 1 μM final concentration of β-amyloid[1-40] for 20 minutes at 25 °C. Intracellular loading of bis-oxonol was attained by incubating the cells with 2 μM bis-oxonol during the last 10 minutes of the 20-minute incubation period with the peptide at 25 °C.

Fluorescence measurements. Flow-cytofluorimetric measurements were carried out at room temperature 22±2 °C. Fluorescence was excited by a Spectra Physics argon-ion laser at 488 nm. The cell-associated fluorescence of 10,000 cells was collected and analysed in a Becton Dickinson FACStaPLUS flow-cytofluorimeter at an emission wavelength of 540±20 nm selected by an appropriate interference filter.

RESULTS AND DISCUSSION

The cells were found to be labelled satisfactorily by passive diffusion on incubation with 2 μM bis-oxonol for 10 minutes at 25 °C. The cells were exposed to a 1 μM final concentration of β-amyloid[1-40] for 20 minutes. The effect of β-amyloid[1-40] peptide on the resting transmembrane potential was monitored by means of comparative cytofluorimetric studies. The flow-cytofluorimetric measurements demonstrated that the distribution of the individual cell-associated probe fluorescence was shifted to lower levels in cells treated with β-amyloid[1-40] for 20 minutes as compared with that of their untreated counterparts (for representative histogram tracings, see Fig. 1). A change in the fluorescence intensity in the same direction was caused by valinomycin, whereas gramicidin induced a shift to higher fluorescence intensities (for representative histogram tracings, see Fig. 2.). Valinomycin is a K^+-specific ionophore known to induce hyperpolarization (Hoffman and Laris, 1974), whereas gramicidin is a channel-forming Na^+/K^+-ionophore causing rapid collapse of the transmentrane potential (Waggoner, 1979).

Table 1. Mean cell-associated fluorescence intensities (FL), standard deviations (S.D.) and numbers of repeated experiments (n) following 20-minute exposures of M26-IF cells to various treatments, including β-amyloid[1-40], valinomycin or gramicidin

	Untreated	ß-[1-40]	Valinomycin	Gramicidin
FL±S.D.	200.9 ± 7.3	174.4 ± 6.9	162.7 ± 7.1	236.4 ± 8.6
(n)	(10)	(6)	(4)	(4)

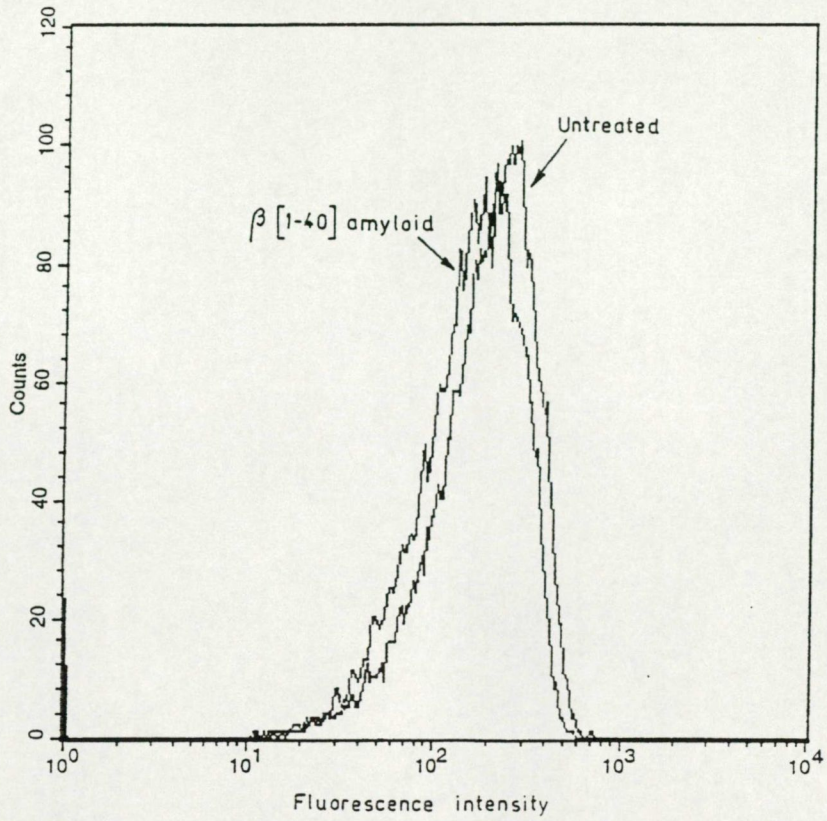

Fig. 1. Representative flow-cytofluorimetric histograms of M26-1F cells treated with 1 μM β-amyloid[1-40] for 20 minutes, during the last 10 minutes of which the cells were loaded with 2 μM bis-oxonol. 10,000 cells were analyzed in each experiment

These findings demonstrate the ability of β-amyloid[1-40] to induce early hyperpolarization in M26-IF cells, an immortalized rat striatal cell line. This observation is in accordance with earlier reports on the action of APP (Furukawa et al., 1996) and β-amyloid peptides on the increased activity of the K^+-channels (Jalonen et al., 1997). This rapid action is one of the earliest cell physiological effects of β-amyloid peptides that has been reported so far. Moreover, the marked similarity of action of β-amyloid[1-140] to that of the K^+-ionophore valinomycin suggests that the early action of β-amyloid peptides might be ionophoric. This finding lends further support to the

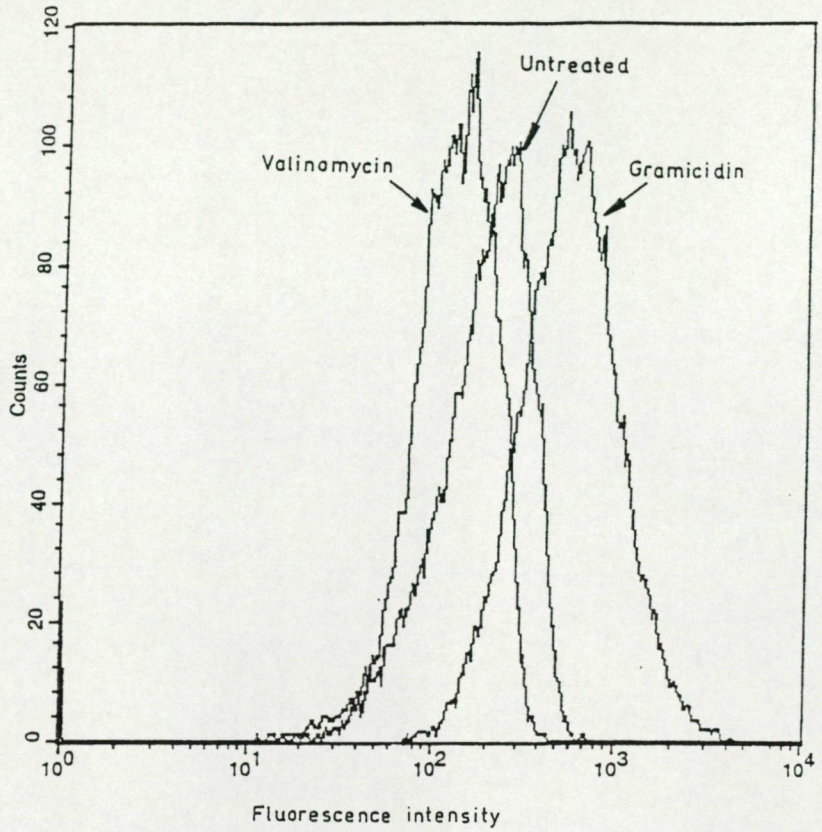

Fig. 2. Representative flow-cytofluorimetric histograms of M26-1F cells treated with 1 μM valinomycin or gramicidin D for 20 minutes, during the last 10 minutes of which the cells were loaded with 2 μM bis-oxonol. 10,000 cells were analyzed in each experiment

documented channel-forming ability of β-amyloid peptides in both model (Mirzabekov et al., 1994) and cellular membranes (Pollard et al., 1995).

Acknowledgements: The M26-IF cell line was a kind gift from the late Dr. Ferenc Joó. This work was supported by grants T017751, T022542 and T022546 from the Hungarian Scientific Research Fund (OTKA), and 80-4/1998/MT from the Ministry of Health. The excellent cell culturing expertise of Mrs. Susan Ambrus is appreciated.

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VI.

Protein and Peptide Letters, Vol. 8, No. 6, pp. 423-428, 2001
Bentham Science Publishers Ltd.
0929-8665/01 $ 20.00 + .00

IN VITRO DEGRADATION OF β-AMYLOID[25-35] PEPTIDE

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Some amyloid-derived peptides show their toxic effects only after a long "aging" period performed several days at pH 7.4 and 37°C for cluster formation. Our experiments show that long aging of β-amyloid[25-35] under physiological conditions results in peptide bond cleavage between Asn^{27} and Lys^{28}. Relatively short incubation time (24 hours) and absence of Ca^{2+} ions prevent peptide cleavage and results in good aggregation of β-amyloids.

INTRODUCTION

A model of the molecular pathomechanism of Alzheimer's disease (AD) involves abnormally elevated concentration of β-amyloid (Aβ) peptides (Aβ[1-40] and Aβ[1-42]) generated by enzymatic cleavage of the β-amyloid precursor protein [1], which induces a cascade of pathological events (increase of Ca^{2+}_i concentration, hyperphosphorylation of tau protein, etc.) in neurons leading to cell death. The "amyloid cascade hypothesis" [2] attributes a central role to Aβ peptides in the pathogenesis of AD. A series of crucial discoveries demonstrated that Aβ peptides exert direct neurotoxic effect *in vitro*, and the undecapeptide fragment Aβ[25-35] shows all the biological effects of the full length peptides [3]. Only the highly aggregated, polymeric forms of Aβ peptides are neurotoxic; monomers show some neuromodulatory effects. "Aging" of Aβ peptides is a common method before *in vivo* experiments [5-7]; this is a long (1-3 days) aggregation process at pH 7.4 for fibril formation. *In vitro* investigations of Aβ neurotoxicity in cell and tissue cultures proceed for several hours or even days [4,8] at pH 7.4, 37 °C. However, these long "aging" and incubation processes could result in degradation for Aβ peptides containing Asn and Met in the peptide sequence. Aβ[25-35] has the following structure:

H-Gly^{25}-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met^{35}-NH_2

The most likely changes under physiological conditions are the well-known spontaneous cleavage of the peptide bond adjacent to Asn [7,9-11], deamidation, isomerization and racemization of Asn as well as oxidation of Met. The aim of our study was to investigate the stability of Aβ[25-35] peptide under physiological conditions simulating the "aging" process and to find an optimal process for Aβ aggregation without deterioration of the peptide structure.

METHODS

Synthesis and purification of amyloid peptides. Aβ[25-35] and its fragments Aβ[27-35], Aβ[28-35], Aβ[25-30], Aβ[25-27], Aβ[30-35] and Aβ[31-35], were prepared by solid-phase methodology on MBHA resin (0.73 mmol/g). Briefly, coupling reactions were carried out with a 3-fold excess of Boc-amino acids with DCC or DCC/HOBt as activating agent, in dichloromethane (DCM) or dimethylformamide (DMF). Intermediate deprotection was achieved with 50% (vol/vol) trifluoracetic acid in DCM containing 0.5% dithiothreitol for 30 min, followed by neutralization with 10% (vol/vol) triethylamine in DCM. After a coupling period of 2 h, the extent of acylation was monitored via the standard ninhydrin test. In the event of incomplete coupling, the coupling procedure was repeated. Final deprotection, and cleavage of the peptides from the resin, were performed with anhydrous hydrogen fluoride in the presence of 8% anisole, 2% dimethyl sulfide, 2% p-cresol, and 2% thiocresol at 0 °C for 60 min. After removal of the hydrogen fluoride under a stream of nitrogen and in vacuum, the free peptides were precipitated with diethylether, filtered off, washed with diethylether, extracted with 50% (vol/vol) aqueous acetic acid, and lyophilized. The synthesized peptides were purified by semi-preparative RP-HPLC and analysed as described earlier [12]. After purification, the purities of the peptides were found to be > 95%. Amino acid analyses of the pure products furnished the expected values.

Peptide incubation. Investigations of the stability of Aβ[25-35] were carried out a) in distilled water; b) under physiological conditions at pH 7.4; and c) in phosphate buffer of pH = 8.0 made with double-distilled and ion-free water in the presence or the absence of Ca^{2+} at 37 °C. The concentration of Aβ[25-35] was in all cases 0.1 mM. The composition of the physiological solution was: NaCl 144.0 mM, KCl 4.0 mM, $CaCl_2$ 1.8 mM, NaH_2PO_4 0.33 mM, $MgCl_2$ 0.53 mM and HEPES buffer 5.0 mM. In order to prevent microbiological degradation, 0.02% NaN_3 was applied in the experiments. The solutions were pipetted into sterilized vials with single-use sterile syringes, and the vials were then sealed. Samples were taken every hour for one day and then daily. The experiment was repeated under an argon atmosphere: before the vials were sealed, argon gas was introduced into the solutions. The reaction was stopped by freezing the samples at –18 °C.

Product analysis. The HPLC analysis of the samples was carried out by using an analytical Knauer HPLC system equipped with a Lichrosorb 5RP-18 (250 x 4 mm) reversed-phase column. The column was eluted with a solvent system consisting of (A) 0.1% aqueous trifluoracetic acid and (B) 0.1% trifluoracetic acid in 80% aqueous acetonitrile in linear gradient mode (0-60% B in 30 min, flow 1.0 ml/min). The eluent was monitored at 220 nm. The different peaks were collected in vials, lyophilized and identified by electrospray mass spectrometry (ES-MS, FinniganMat TSQ 7000 mass spectrometer).

RESULTS

Stability investigations were carried out under physiological conditions in the presence or absence of Ca^{2+}. Each experiment was repeated three times. The rates of degradation in the repeated experiments were similar. The possibility of biological degradation can be precluded because of the use of NaN_3 and sterilized water in sterile and sealed vials.

The investigations were first performed only in distilled water and under physiological ion conditions at 37 °C in the presence of Ca^{2+}. Samples were taken hourly for 24 h, frozen and analysed by RP-HPLC. It was found that, in distilled water at 37 °C, Aβ[25-35] was stable without any signs of decomposition, even after 5 days (Fig. 1).

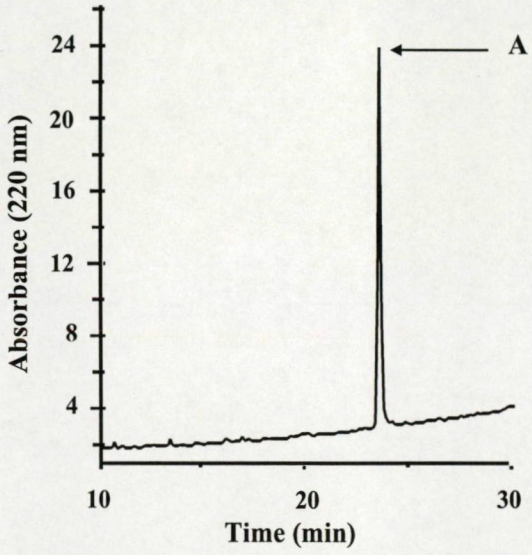

Figure 1. HPLC-chromatogram of βA[25-35] (denoted by letter A) after a 5-day incubation in distilled water.

At physiological ion concentration and in buffer solution of pH 7.4, Aβ[25-35] was stable during the first 12 h at 37 °C, but after 19 h a new peak appeared in the RP-HPLC chromatogram (Fig. 2, left panel). After 5 days, more than 50% of the peptide was decomposed (Fig. 2, right panel).

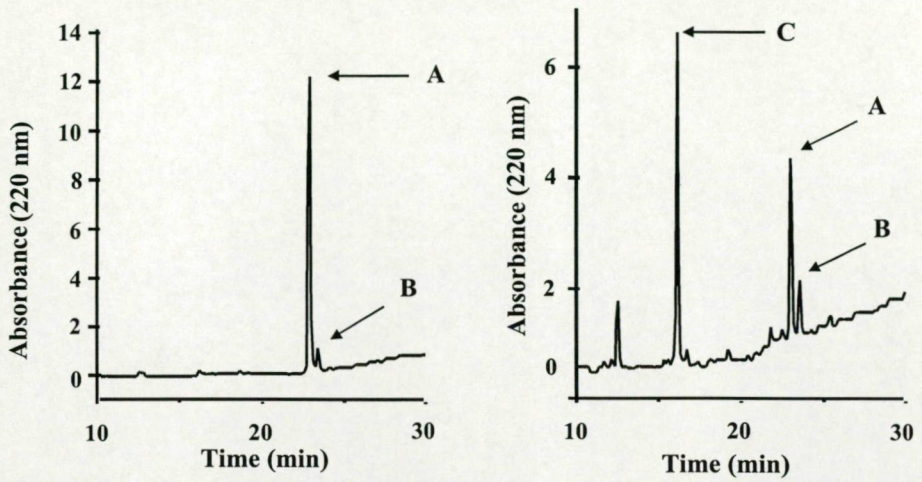

Figure 2. HPLC-chromatograms of Aβ[25-35] after 19 hours (left panel) and after 5 days (right panel) under physiological conditions. Aβ[25-35] peptide is denoted by letter A, Aβ[28-35] peptide by letter B and Aβ[28-35](Met-O) by letter C.

The peaks from the analytical HPLC column were isolated and lyophilized, and their R_f values were compared with those of the following synthetic fragments of Aβ[25-35]: Aβ[27-35], Aβ[28-35], Aβ[31-35] and Aβ[25-30]. These fragments were synthesized as reference standards for HPLC and Ms (Table 1). The presence of the peptide fragment βA[28-35] (M_w = 800) and the absence of the fragments Aβ[27-35], Aβ[31-35] and Aβ[25-30] were demonstrated. Mass spectrometry revealed the Ca^{2+} salts of the peptides Gly-Ser-Asn-OH and Gly-Ser-Asp-NH_2 (identical molecular weight: M_w = 276), Aβ[28-35] and the oxidized form of Aβ[28-35] (M_w = 816).

At pH 7.0 only slight decomposition was observed. Experiments were also carried out at pH 8.0 in phosphate buffer, both in the presence and in the absence of Ca^{2+}. The results indicated that the degradation process accelerates at pH > 7.0 and in the presence of Ca^{2+}.

Table 1. Synthesized peptides used in the study

Sequences	Mass spectra	
	Calculated (average mass)	Found
Aβ[25-35]-NH_2	1059.29	1059.0
Aβ[28-35]-NH_2	801.06	800.0
Aβ[27-35]-NH_2	915.16	915.0
Aβ[25-30]-NH_2	531.57	531.0
Aβ[25-30]-OH	532.57	532.0
Aβ[30-35]-NH_2	615.83	615.0
Aβ[31-35]-NH_2	544.76	544.0
Aβ[25-27]-NH_2	275.22	275.0

Stability investigations under argon atmosphere were performed in the absence of Ca^{2+}. The results suggest that the oxidation of Met can be avoided under these conditions. Degradation of the peptide chain occurs at only a very low rate (Fig. 3).

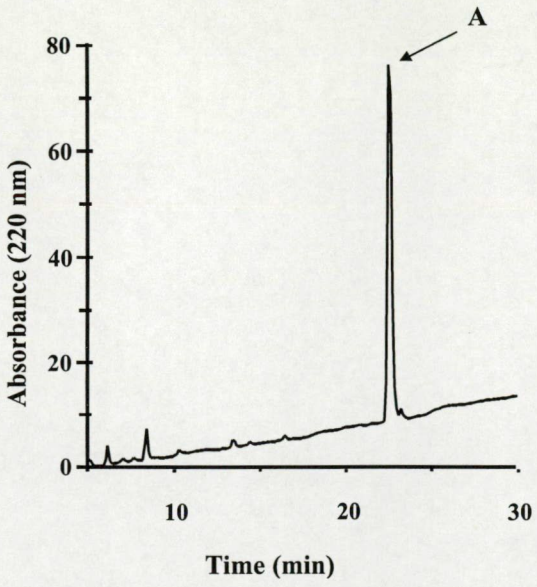

Figure 3. HPLC-chromatogram of Aβ[25-35] (denoted by letter A) after storage for 52 hours under an argon atmosphere at pH=8.0, in the absence of Ca^{2+}.

DISCUSSION

Our studies show that Aβ[25-35] is not stable under physiological conditions: the peptide chain is hydrolysed at Asn^{27} resulting in isomer tripeptide fragments (Aβ[25-27] with Asn or isoAsn at the C-terminal end) and the octapeptide Aβ[28-35]. The cleavage mechanism may involve formation of succinimide ring as described for other peptides [13,14]. It also is affected by the presence of Ca^{2+} ions and increased Ca^{2+}-complexation facilitates degradation and peptide bond cleavage. It is interesting that this Asn-Lys sequence has not been described in the literature for being sensitive to hydrolysis under mild conditions. As Aβ[25-35] undergoes a relatively rapid decomposition at pH 7.4 if the buffer contains Ca^{2+} ions, long incubation times and "aging" processes of Aβ peptides should be avoided. According to our results, the method of choice would be a relatively short (24 hours) incubation of Aβ[1-42] [5] at pH 7.4 in aqueous solution, but without Ca^{2+} ions and under argon atmosphere in order to avoid oxidation of Met^{35}.

ACKNOWLEDGEMENTS

This work was supported by grants from the Hungarian National Research Foundation (OTKA T-22546 and T-17751).

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Received on May 2, 2001, accepted on July 2, 2001

VII.

Naunyn-Schmiedeberg's Archives of Pharmacology

Supplement Number 1 to Volume 356 No. 4, 1997

Abstracts of the Fall Meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie

(with the participation of the Pharmacological Societies of Austria, The Czech Republic, Hungary, The Slovak Republic, and Slovenia)

14–17 September 1997, Wien, Austria

Contents

Springer
210 Naunyn-Schmiedeberg's Arch Pharmacol
ISSN 0028-1298 NSAPCC 354 (Suppl. 1) R1–R64 (1997)

141

CHEMICAL DEGRADATION OF β-AMYLOID [25-35] PEPTIDE UNDER PHYSIOLOGICAL CONDITIONS

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In the pathogenesis of Alzheimer-disease a neurotoxic peptide, called β-amyloid (βA), has the fundamental role. This peptide is 40 to 42 amino acids long and it is formed by proteolytic cleveage from the amyloid precursor protein (APP). The [25-35] undecapeptide fragment proved to be the active centre of the full-length βA peptide. This fragment is often used for biological experiments, because it is substantially cheaper and easily preparable, in comparison with the 40- and 42-mers.

In the in vitro biological experiments, the cell cultures are tipically incubated for days at 37°C with βA peptides. In some experiments preaged solutions are used. We were interested whether aging of peptides under these conditions includes not only conformational change, but also chemical modifications. For this end we investigated the stability of βA [25-35] in distilled water, and in a buffer on pH = 7.4 and physiological ion concentrations, at 37°C. In distilled water and at 37°C the peptide was stable without any signs of decomposition even after a 3-days storage.

In the buffer solution βA [25-35] showed a marked decomposition with predominant cleavage of the peptide chain between Asn^{27} and Lys^{28}; oxidation of Met^{35} also occured. After a 3-days storage 50-60% of the peptide was decomposed, indicating a potential role of chemical modifications in the aging-related change of the biological activity of βA.

VIII.

PROCEEDINGS OF THE
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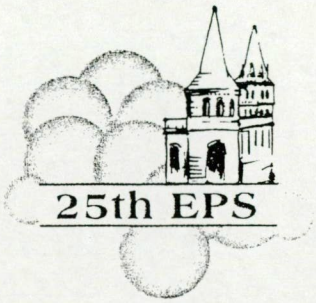

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Stability Studies on β-Amyloid [25-35] Peptide

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Introduction

The Alzheimer's disease is a neurodegenerative disorder of the central nervous system. One of the major characteristics of the disease is the excessive accumulation of β-amyloid peptide (βA) in senil plaques, neurofibrillary tangles and cerebrovascular deposits found in the brain of patients with Alzheimer's disease [1]. It has been reported that βA and βA fragments show neurotoxic effects in the cortex, hippocampus, amygdala and cholinergic system [2]. The effects of βA are localized to amino acid residues 25-35 (βA[25-35]) of the full-length peptide [3].

In the *in vitro* experiments under physiological conditions, the cell cultures are incubated with the βA and βA pepide fragments at 37°C for several hours or days [4]. In some *in vivo* experiments preaged solutions are used [5]. In this work, we report the examination of the stability of βA[25-35] under conditions used in the biological experiments.

Results and Discussion

The stability of βA[25-35] was investigated in distilled water and in buffers at physiological, neutral, and basic pH at 37°C. In order to guarantee the steril circumstances, all our experiments were performed in sterilized and then sealed vials in the presence of NaN_3. After the stability experiments, the solutions were checked and found sterile.

In distilled water at 37°C, βA[25-35] was stable without any signs of decomposition even after a three-days storage.

In case of physiological ion concentration, in pH=7.4 buffer solutions, some decomposition and the presence of βA[28-35] fragment can be observed after 19 hours. After a 4-days storage under the above conditions, more than 50% of the peptide was decomposed.

The stability investigation was also carried out at pH=7.0, and a slight decomposition was found.

At pH=8.0, the experiments were carried out either in the presence or in the absence of Ca^{2+}. According to our results, the cleavage occurs faster in the presence of Ca^{2+} (Figure 1.). The supposed cleavage mechanism of the peptide chain which occurs through a transitional succinimid state at Asn^{27} [6]. The presence of βA[28-35] peptide resulted by the cleavage was demonstrated by HPLC and electrospray mass-spectrometry.

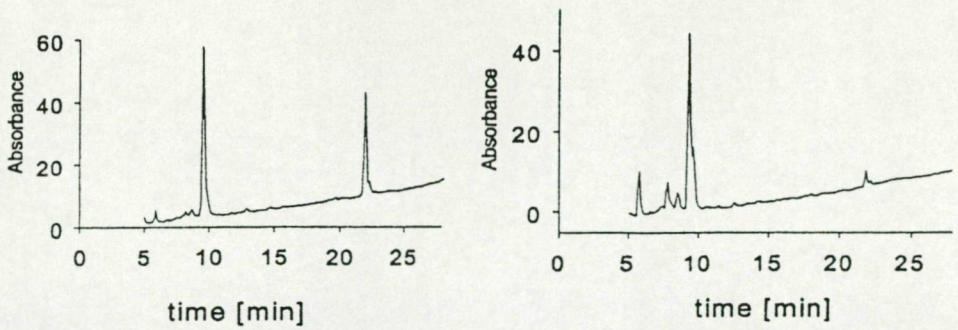

Figure 1. *HPLC chromatogram after 66 hours at pH=8.0 in absence of Ca^{2+} (left panel) and in presence of Ca^{2+} (right panel). R_t(βA[25-35]) = 21.9 min*
HPLC conditions: Knauer HPLC system, column: LiChrospher WP300 5RP-18; gradient: 10 → 45% B (B = 80% ACN, 0.1% TFA)

Acknowledgements

This work was supported by OTKA Grants # T-22546 and T-17751.

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